Bypassing pan-enterovirus host factor PLA2G16

Jim Baggen1,4,5, Yue Liu2,4,5, Heyrhyoung Lyoo1, Arno L.W. van Vliet1, Maryam Wahedi1, Jost W. de Bruin1, Richard W. Roberts1, Pieter Overduin3, Adam Meijer3, Michael G. Rossmann2, Hendrik Jan Thibaut1,4,5 & Frank J.M. van Kuppeveld1,4,5

Enteroviruses are a major cause of human disease. Adipose-specific phospholipase A2 (PLA2G16) was recently identified as a pan-enterovirus host factor and potential drug target. In this study, we identify a possible mechanism of PLA2G16 evasion by employing a dual glycan receptor-binding enterovirus D68 (EV-D68) strain. We previously showed that this strain does not strictly require the canonical EV-D68 receptor sialic acid. Here, we employ a haploid screen to identify sulfated glycosaminoglycans (sGAGs) as its second glycan receptor. Remarkably, engagement of sGAGs enables this virus to bypass PLA2G16. Using cryo-EM analysis, we reveal that, in contrast to sialic acid, sGAGs stimulate genome release from virions via structural changes that enlarge the putative openings for genome egress. Together, we describe an enterovirus that can bypass PLA2G16 and identify additional virion destabilization as a potential mechanism to circumvent PLA2G16.
The Enterovirus genus (family Picornaviridae) comprises a large group of non-enveloped positive-stranded RNA viruses harboring 13 species, of which seven contain human pathogens (Enterovirus A–D and Rhinovirus A–C). Due to the absence of an envelope, enteroviruses need to deliver their genome across a membrane to infect a cell. Following receptor binding and endocytosis, virus particles uncoat by forming a proteinaceous pore spanning the endosomal membrane through which they eject their genome into the cytosol. The uncoating process is triggered by cellular uncoating cues (receptor binding or low endosomal pH), which promote virion conversion into an expanded state (A-particle) that favors membrane interaction. Most enteroviruses, such as poliovirus and coxsackievirus B3 (CV-B3), require only their receptor for uncoating, whereas other viruses, like enterovirus A71 (EV-A71), also require acidification of the endosomal lumen. Minor-group rhinoviruses (including rhinovirus A2 (RV-A2)) are exceptional, as these do not engage an uncoating receptor but uncoat exclusively via endosomal acidification. Uncoating receptors bind to a depression on the outer surface of the virus called the canyon and interact with the VP1 GH loop, thereby inducing the displacement of a stabilizing lipid, the pocket factor, from a hydrophobic pocket below the canyon.

Recently, using haploid genetic screens, PLA2G16 was identified as an enterovirus host factor. PLA2G16 is a lipid-modifying enzyme implicated in adipocyte lipolysis. Knockout of PLA2G16 did not affect virus binding to the cell surface, endocytic uptake, translation or replication but hampered the dissociation of genomic RNA from virus-containing endosomes. This showed that PLA2G16 promotes the timely delivery of the viral genome to the cytosol, before the virus-induced membrane permeation is detected by the cellular sensor galectin 8 and the virus is cleared by the autophagy machinery. The mechanism by which PLA2G16 facilitates viral genome delivery remains to be established.

EV-D68 is an atypical enterovirus that usually causes mild respiratory tract disease, but is also associated with severe lower respiratory tract infections and acute flaccid myelitis. Because EV-D68 used to be a rare pathogen, it was poorly studied for a long time. However, the increased incidence of EV-D68-associated illness has accelerated research on this virus. Using a genome-wide haploid genetic screen with the EV-D68 prototype strain Fermon, we previously identified sialic acid (Sia) as an essential host factor and showed that both a2,3- and a2,6-linked Sia can serve as receptors. Moreover, crystallography revealed a Sia-binding site in the canyon and showed that Sia binding dislodges the pocket factor, suggesting a role of Sia in facilitating uncoating. However, no A-particle formation or genome release was observed upon incubation with Sia. Another study identified the neuron-specific protein intercellular adhesion molecule 5 (ICAM-5) as an EV-D68 receptor and showed that glycosylation at residue Asn94 in ICAM-5 is required for virus binding. ICAM-5 was found to promote the transformation of mature virions to A-particles and to facilitate genome release in vitro, implicating ICAM-5 as an EV-D68 uncoating receptor. Additionally, it was recently shown that EV-D68 infection of cells also requires endosomal acidification and that acid treatment induces A-particle formation in vitro. It is currently unknown how ICAM-5, Sia, and endosomal acidification cooperate to destabilize EV-D68 virions.

Although Sia is an essential receptor for most strains, we previously identified several EV-D68 clinical isolates, including EV-D68-947, that were able to infect Sia-deficient HAP1 SLC35A1KO cells, indicating that these strains can use alternative receptors. In this study, we set out to identify such alternative receptors and show that, in the absence of sialylated glycans, EV-D68-947 can employ sulfated glycosaminoglycans (sGAGs). We reveal that this dual receptor-binding strain requires PLA2G16 only when infecting cells via Sia, but not when using sGAGs. Using cryo-EM analysis, we show that sGAG binding induces structural rearrangements in the viral capsid and stimulates genome release in vitro. Our findings suggest a role of PLA2G16 as the last in a series of uncoating cues and point towards additional virion destabilization as a mechanism to circumvent the pan-enterovirus host factor PLA2G16.

Results
Both Sia and sGAGs can serve as EV-D68-947 receptors. To ensure exclusive usage of non-sialylated receptors, we performed a haploid genetic screen with EV-D68-947 in HAP1 SLC35A1KO cells, which lack surface-expressed Sia. This screen identified many genes related to the synthesis of sGAGs, which are long, unbranched, highly sulfated polysaccharides. Hits include genes involved in synthesis of the GAG core tetrasaccharide (B3GAT3, FM20B, B3GALT6, B4GALT7, UXS1, and XYL2), elongation of heparan sulfate (EXT1, EXT2, and EXT3), synthesis of UDP-glucuronate (UGP2, UGDH) and sulfation (SLC35B2, NDST1). Together, these hits pointed towards a role of sGAGs as an alternative receptor for EV-D68-947. To confirm that EV-D68-947 binds to sGAGs, we performed neutralization experiments using various soluble sGAG analogues. Virus incubation with soluble heparin or low-molecular-weight-heparin (LMWH) neutralized infection with EV-D68-947, while the Sia-dependent EV-D68-Fermon was not neutralized.

PLA2G16 has been identified as a host factor in several enterovirus infections. PLA2G16 was observed in the screen with EV-D68-947, and the virus is cleared by the autophagy machinery. The mechanism by which PLA2G16 facilitates viral genome delivery remains to be established.

Engagement of sGAGs reduces the PLA2G16 dependency of EV-D68. PLA2G16 has been identified as a host factor in several enterovirus haploid genetic screens, including a screen with EV-D68-Fermon. In contrast to these screens, no significant enrichment of disruptive gene-trap insertions in PLA2G16 was observed in the screen with EV-D68-947 in SLC35A1KO cells, suggesting that this strain has unusual...
properties that eliminate the need for this host factor under certain conditions. EV-D68-947 originated from a patient with mild respiratory tract disease and was isolated by two passages in rhabdomyosarcoma (RD) cells. Deep sequencing analysis revealed that two substitutions in capsid proteins VP2 and VP1 were introduced during isolation (Supplementary Fig. 2a). To validate this, we generated an infectious cDNA clone in which the original genome was restored and compared this virus (947) with EV-D68-2042 (2042-4/5/6/7) enabling this virus to infect HAP1 CMASKO cells, another Sia-deficient cell line. Introduction of a subset of these residues was tolerated for some combinations and led to reversion for other combinations. Mutant 2042-4/7, containing residues VP3 Glu59 and VP1 Lys271, could infect HAP1 CMASKO cells for other combinations. Mutant 2042-4/7, containing residues VP3 Glu59 and VP1 Lys271, could infect HAP1 CMASKO cells for other combinations. Mutant 2042-4/7, containing residues VP3 Glu59 and VP1 Lys271, could infect HAP1 CMASKO cells for other combinations. Mutant 2042-4/7, containing residues VP3 Glu59 and VP1 Lys271, could infect HAP1 CMASKO cells for other combinations.

Both sialic acid and sulfated glycosaminoglycans can serve as EV-D68-947 receptors. A haploid genetic screen for host factors of EV-D68-947. Each circle represents a gene, with size corresponding to the number of genetraps inserted per gene. The y-axis indicates the significance of enrichment of insertions in a gene, compared to an uninfected control population. Genes were randomly distributed on the x-axis. B Schematic overview of sulfated glycosaminoglycan (sGAG) synthesis, showing the functions of genes identified in the haploid screen, which are indicated in italics. UTP, uridine-5'-triphosphate; UDP, uridine diphosphate; P, phosphate; Glc, glucose; GlcA, glucuronic acid; IdoA, iduronic acid; GlcNAc, N-acetylglucosamine; Gal, galactose; Xyl, xylose. C EV-D68 strains were incubated with various concentrations of heparin or low-molecular-weight-heparin (LMWH), followed by infection with HeLa-R19 cells, dsRNA staining and quantification of infected cells. Two technical replicates are shown. D HeLa-R19 cells were treated with neuraminidase (NA), sodium chlorate (NaClO3) or a combination of both and infected with EV-D68, followed by staining of dsRNA (green) and nuclei (blue). Shown are representative confocal micrographs. Values denote the number of infected cells (mean ± s.e.m. of 3–4 technical replicates) as percentage of mock. Scale bar: 150 μm. The experiment was conducted three times with similar results.
EV-D68-947 acid independency and ICAM-5 binding properties. Although sGAG usage reduced the PLA2G16 dependency of EV-D68-947 (Fig. 2e), this mutant was more dependent on PLA2G16 than EV-D68 (947) (Fig. 2e), suggesting that additional factors influence the level of PLA2G16 requirement during virus entry. To explore whether EV-D68 strains require different uncoating cues, we compared sensitivity to the V-ATPase inhibitor Bafilomycin A1 (BafA1). Virus production of EV-D68 strains Fermon and 2042 was inhibited by BafA1, indicating that these strains, like the positive control RV-A25, rely on endosomal acidification during uncoating (Supplementary Fig. 3a). Similarly, analysis of the number of infected cells showed that the gain-of-function mutant 2042-4/7 was BafA1-sensitive (Fig. 3a), whereas BafA1 treatment hardly affected the negative control CV-B3 and EV-D68-947.
and stained for dsRNA, followed by quantification of infected cells. The experiment was conducted twice with similar results. **b** Viruses were incubated at 48 °C for the indicated times, followed by infection of HeLa-R19 cells, dsRNA staining and quantification of infected cells. Four technical replicates with a fitted sigmoidal curve are shown. **c** Viruses were incubated with soluble ICAM-5 (sICAM-5), followed by infection of HeLa-R19 cells, dsRNA staining and quantification of infected cells. The experiment was conducted twice with similar results. **d** WT or ICAM-5 KO HAP1 cells were infected and stained for dsRNA, followed by quantification of infected cells. Error bars represent the mean ± s.e.m. of three (a, b) or four (c, d) technical replicates.

**A basic patch in EV-D68-947 as putative sGAG binding site.** To investigate whether sGAGs bypass PL2G16 by destabilizing the EV-D68-947 particles, we studied the effects of different glycan receptor analogues on virion structure by cryo-EM. Structures of EV-D68-947 were determined after incubation of the virus either in the absence of a receptor analogue, with 6′SLN (a sialylated trisaccharide), with dp6 (a heparin-derived hexasaccharide), or with LMWH at 33 °C (Supplementary Figs. 4 and 5 and Supplementary Table 1). A 2.4 Å resolution structure of the virus in complex with 6′SLN showed that EV-D68-947 binds to Sia in nearly the same way as does EV-D68-Fermon, despite some differences in the amino acids near the receptor-binding site (Fig. 4a, b). We did not observe density corresponding to dp6 or LMWH in the EV-D68-947 structure, either because these ligands, in contrast to Sia, interact with the virus via a multitude of low-affinity and/or transient electrostatic interactions or because the bound ligands do not occupy all icosahedral symmetry related sites. Nevertheless, the structure of the EV-D68-947 native virion showed a basic patch (formed by VP1 residues Lys268, Lys270, Lys271, and Arg272) that is not present in EV-D68-Fermon (Supplementary Fig. 6a). This basic patch presumably serves as a binding site for the negatively charged sGAGs and contains the positively charged Lys at VP1 position 271, which conferred a sGAG-binding capacity to mutant 2042-4/7 (Fig. 2c, Supplementary Fig. 6b). Together, these data point towards putative sGAG-binding residues in EV-D68-947 near the Sia-binding site.

**sGAG analogues stimulate EV-D68-947 uncoating.** Engagement of sGAGs, but not Sia, resulted in a reduced PL2G16 dependency (Fig. 2b). Structural analyses showed that, like 6′SLN, both sGAG analogues induced movements of the VP1 GH loop into the VP1 hydrophobic pocket, causing displacement of the pocket factor (Fig. 4c), as previously observed. This indicates that both Sia and sGAGs can prime the uncoating process. By contrast, determination of the ratio between the number of EV-D68-947 full and empty particles after 1 h incubation at 33 °C showed that the sGAG analogues dp6 and LMWH, but not 6′SLN, promoted genome release (Fig. 4d and Supplementary Fig. 7a–d). Structural comparison of LMWH-induced empty particles with empty particles formed in the absence of a ligand showed that LMWH caused an enlargement of the openings in the capsid around the two-fold symmetry axes that serve as putative sites of genome egress (Fig. 4e and Supplementary Fig. 7e). Together, these data demonstrate that sGAGs, but not Sia, stimulate genome release from EV-D68-947, providing a...
possible explanation for the capacity of EV-D68-947 to bypass PLA2G16 when infecting cells via sGAGs.

**PLA2G16 evasion via additional virion destabilization.** Our findings may be explained by a model in which subsequent uncoating cues orchestrate the priming of the EV-D68 particle for RNA release (Fig. 4f). The catalytic activity of PLA2G16 may constitute the ultimate event that triggers release of the genome.

Requiring such a host factor as a final checkpoint for RNA release might be beneficial for the virus, as it postpones genome release until the appropriate intracellular compartment has been reached. sGAGs binding, which is not strictly required for EV-D68-947 infection, may constitute an unnecessary destabilizing force that causes more extensive destabilization than is minimally required to prime the virion for PLA2G16-mediated infection. Such additional virion destabilization could disturb the delicate balance of uncoating cues and enable the virus to release its genome.
before encountering PLAG216. Whether the alternative mode of endocytic uptake (clathrin-independent) via sGAGs (Supplementary Fig. 1c) is an additional factor influencing PLAG216 (in) dependency, e.g. by altering the microenvironment in which uncoating takes place, is unknown. To investigate whether a link between virion stability and PLAG216 dependency is unique for EV-D68 or also applies to other enteroviruses, we passaged EV-A71 (a member of the enterovirus A species) in PLAG216-deficient knockout cells. This resulted in a mutant (passage 5) with multiple capsid substitutions (Supplementary table 2) that displayed a reduced PLAG216 dependency (Fig. 4g). Concomitantly, this EV-A71 mutant was less thermostable than the parental virus (Fig. 4h), providing an additional line of support for a link between enterovirus stability and PLAG216 dependency.

Discussion

PLA2G16 was identified as an enterovirus host factor that facilitates the timely delivery of viral RNA into the cytoplasm before the virus is cleared by autophagy. This enzyme is a promising target for broadly acting antiviral drugs, since it is required by all human enterovirus species tested thus far. We previously identified Sia as an EV-D68 receptor and showed that several strains, including EV-D68-947, can infect Sia-deficient cells. In this study, we showed that EV-D68-947 is a dual receptor-binding virus that can use both sialylated glycans and sGAGs as receptors. Interestingly, we found that EV-D68-947 only requires PLA2G16 when infecting cells via Sia, but not via sGAGs. Consistently, introduction of a sGAG-binding site into the Sia- and PLA2G16-dependent strain EV-D68-2042 reduced its PLA2G16 dependency, although not to the same extent as EV-D68-947. We showed that this relative PLA2G16 independency of EV-D68-947 coincides with an enhanced sensitivity to neutralization by soluble ICAM-5, indicative of an enhanced or more disruptive interaction with this receptor. In addition, we showed that sGAGs stimulate EV-D68-947 genome release in vitro. While most enterovirus uncoating receptors are essential uncoating cues, sGAGs are not required for EV-D68-947 uncoating per se, but probably form an extra layer of destabilization that disturbs the natural balance of uncoating cues. Together, these results suggest that PLA2G16 independency is enabled by additional receptor-mediated virion destabilization. Finally, the observation that EV-A71 could adapt to replication in the absence of PLA2G16 via mutations that reduced virion stability (Fig. 4g, h) further implicates additional virion destabilization as a mechanism to bypass PLA2G16.

Structural analysis showed that Sia binds to EV-D68-947 in the same site as in EV-D68-Fermon and induces pocket factor displacement. Incubation of EV-D68-947 with sGAG analogues not only caused pocket factor displacement but also promoted genome release, by enlarging the putative openings for genome egress at the two-fold axes via a yet unknown mechanism. Unfortunately, we were unable to determine the exact binding site of sGAGs on the EV-D68-947 particle by cryo-EM. Nevertheless, introducing two surface residues into EV-D68-2042 (Fig. 2c) conferred a sGAG-binding capacity and pointed towards putative sGAG-binding residues in a basic patch near the Sia-binding site, which is in a similar location as the sGAG-binding site in FMDV. One of these putative GAG-binding residues, VP3 Glu27, is present in most other published EV-D68 strains, whereas VP1 Lys278 is absent in other clade B strains and rarely occurs in other clades. Analysis of viral sequences in the clinical sample from which EV-D68-947 was isolated, revealed that the isolated virus had adapted to cultured cells via two capsid substitutions, including VP1 Lys278. Hence, it remains to be established whether EV-D68-947 originally possessed a sGAG-binding capacity or acquired this property during cell passaging. Future research should determine whether sGAGs serve as EV-D68 receptors in vivo, by studying non-cell-adapted strains in physiologically relevant models like human airway epithelial (HAE) cultures.

In addition to glycan receptors, EV-D68 engages the cell-surface protein ICAM-5 as a receptor that promotes virus uncoating. In this study, we confirmed the role of ICAM-5 as an EV-D68 receptor by showing that ICAM-5 knockout reduces infection by EV-D68 strains Fermon, 947, and 2042, even though this receptor was not essential for infection (Fig. 3d and Supplementary Fig. 1d). Although ICAM-5 knockout caused a similar reduction in infection for these strains, we found that soluble ICAM-5 has a different neutralizing effect on EV-D68-947 than on EV-D68-2042 and mutant 2042-4/7 (Fig. 3c). This may be due to an enhanced interaction with ICAM-5 or, alternatively, ICAM-5 may have a different destabilizing effect on EV-D68-947. Because the EV-D68 capsid residues directly involved in ICAM-5 binding are unknown, structural studies are needed to elucidate the details of this interaction. Such studies may also shed more light on the possible link between the neuron-specific protein ICAM-5 and neurological disease induced by different EV-D68 strains.

Together, our data reveal an unexpected connection between receptor binding and the requirement of PLA2G16 in cells, representing the first and last steps of enterovirus entry, respectively. This suggests that PLA2G16 plays a role in a virus-associated entry step, such as transmembrane pore formation or RNA translocation across the membrane. PLA2G16 might serve to initiate pore formation, improve pore permeability, or enhance
Methods

**Cells and viruses.** HAP1, HAP1 CMAS KO, and HAP1 ICAM-5 KO cells were obtained from Horizon Discovery Group plc (Cambridge, UK). HAP1 B3GALT6 KO, HAP1 SLC35A1 KO, H1-HeLa, and H1-HeLa PLA2G16 KO cells were obtained from Thijn Brummelkamp (Netherlands Cancer Institute, Amsterdam). HeLa R19 cells were obtained from G. Belov (University of Maryland and Virginia-Maryland Regional College of Veterinary Medicine, US). HuH7/Lunet/T7 cells were obtained from Ralf Bartenschlager (Heidelberg University Hospital, Germany). Rhabdomyosarcoma (RD) cells were obtained from the European Collection of Cell Cultures (Catalogue No: 85111502). HAP1 cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM, Lonza) containing 10% (v/v) fetal calf serum (FCS). HeLa, HuH7/Lunet/T7, HEK293T (ATCC CRL-11216) and RD cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Lonza) containing 10% (v/v) FCS. All cell lines tested negative for mycoplasma contamination.

**Infectivity assays.** Cells were infected with the virus using a multiplicity of infection (MOI) of about 0.01. At about 60 h post infection, cells and supernatant were harvested and separated by centrifugation. The pellets were subjected to multiple cycles of freezing and thawing, followed by removal of cell debris via centrifugation. All supernatant was combined and spun down at 278,000 x g (Ti 50.2 rotor) for 2 h. The resultant pellets were resuspended in 250 mM HEPES, 250 mM NaCl, pH 7.5 (buffer 1), and sequentially treated with MgCl2, NaN3, and tyramide signal amplification (TSA) solution (pH 9.5), and sodium n-lauryl-sarcosinate with a final concentration of 5 mM, 10 µg mL⁻¹, 0.25 mg mL⁻¹, 0.8 mg mL⁻¹, 15 mM, and 1% (w/v), respectively. The resultant sample was spun down, yielding pellets that were then resuspended in buffer 1. The crude virus was purified through a potassium tartrate density gradient (10–40% (w/v)) using a SW 41 Ti rotor. Purified virus was stored in phosphate buffer saline at 4 °C. All other virus stocks were produced by propagating virus in HeLa-R19 cells, subjecting cells and supernatant to three cycles of freezing and thawing, and centrifugation to remove cell debris.

**Cryo-electron microscopy.** For structure determination, purified EV-D68 947 was treated independently with 10 mg mL⁻¹ 6 SLN for 30 min (dataset virus-6 SLN), with 5 mg mL⁻¹ 6 SLN (dataset virus-5 mLW, with 5 mg mL⁻¹ dp6 for 1 h (dataset virus-dp6), or without a receptor analogue for 30 min (dataset virus-alone). These treatments were all at 33 °C. Aliquots of 2.7 µL of the resultant sample was applied onto copper grids with a continuous Lacey carbon film (400 mesh, Ted Pella). After blurring for about 5 s, the grid was plunge frozen into liquid ethane using a Cryoplunge 3 system (Gatan). Movies of EV-D68 particles embedded in a thin layer of vitreous ice were imaged on an FEI Titan Krios transmission electron microscope operated at 300 kV and equipped with a K2 Summit direct electron detector (Gatan). Data were automatically collected using the program Leginon (31). The dose rate was about 4 e⁻ pixel⁻¹ s⁻¹ for dataset virus-6 SLN and 8 e⁻ pixel⁻¹ s⁻¹ for all other datasets. Each view was collected at a nominal magnification of ×22500 in electron counting mode with defocus ranging from 0.5 to 3.1 µm. This gave a pixel size 1.30 Å pixel⁻¹ at the specimen level. A total electron dose of 33 e⁻ Å⁻² was fractionated into 28 frames with a frame rate of 250 ms frame⁻¹. Datasets virus-dp6 and virus-LMWH were collected at a nominal magnification in super resolution mode, giving a super resolution pixel size of 0.65 Å pixel⁻¹. The defocus range was 0.7–5.4 µm (dataset virus-dp6) and 0.6–4.5 µm (dataset virus-LMWH). A total electron dose of 33 e⁻ Å⁻² was fractionated into 35 frames with a frame rate of 200 ms frame⁻¹. Dataset virus-6 SLN was collected at a nominal magnification of ×29000 in super resolution mode with a defocus range of 0.4–3.6 µm. This gave a super resolution pixel size of 0.30 pixel⁻¹. A total electron dose of 25 e⁻ Å⁻² was fractionated into 62 frames with a frame rate of 100 ms frame⁻¹.

To test the effect of receptor analogues on viral stability, purified EV-D68 947 was incubated with 10 mg mL⁻¹ 6 SLN, 5 mg mL⁻¹ dp6, or 5 mg mL⁻¹ LMWH for 2 h. These complexes were formed at ligand–virus molar ratios of 1:1 and 1:2. After incubation, the samples were subjected to electron microscopy. A total of 8,000 particles were collected at 300 kV and each particle was assigned a super resolution score. The super resolution scores were used to calculate the number of particles with a super resolution score higher than 0.5. Dataset virus-6 SLN was collected at a nominal magnification of ×29000 in super resolution mode with a defocus range of 0.4–3.6 µm. This gave a super resolution pixel size of 0.30 pixel⁻¹. A total electron dose of 25 e⁻ Å⁻² was fractionated into 62 frames with a frame rate of 100 ms frame⁻¹.
8.6 × 10^11. As a control, virus was incubated in the absence of a receptor analogue. Cryo-EM sample preparation was performed as described above. Electron micrographs were manually collected at a nominal magnification of ×29500 using a total electron dose of ~25 e− Å^−2 on an FEI Phillips CM200 TEM operated at 200 kV and equipped with a 4 k × 4 k charged coupled device (Gatan). The defocus ranged from 0.7 to 6.0 μm. Particles were selected using e2boxer.py (part of the EMAN2 program package38), as will be detailed below. The number of full and empty particles was counted by three individuals. The total number of particles used for counting was 3690 (virus alone), 3023 (virus with d6p), 3107 (virus with LMWH), or 3492 (virus with 6’SLN).

Image processing. Movie frames were aligned using the program MotionCor235. In this process, the first frame that had a large motion was discarded, and a reported weighting scheme was employed to down-weight the high resolution information in late frames. The aligned frames were summed up and binned by a factor of two to yield individual electron micrographs. Binning was performed for all datasets except for dataset virus-alone at this stage. The non-dose-weighted micrographs were utilized to estimate contrast transfer function (CTF) parameters using the program CTFFIND47. Projections derived from a three-dimensional (3D) reconstruction of EV-D68 strain US/MO14-18947 were low pass filtered to 40 Å resolution and served as templates for particle selection from the dose-weighted micrographs using e2boxer.py49. Particle images were then extracted and subjected to two-dimensional classification using the program Relion28. This procedure allowed for separation of full and empty particles and for removal of low-quality particles.

Samples of EV-D68 947 contained a mixed population of full particles after about 2 weeks of storage at 4 °C. For datasets virus-alone and virus-d6p, images of full particles (5.20 Å pixel^−1) were subjected to 3D classification using the program Relion, in whichicosahedral symmetry was imposed. This process enabled selection of a subset of particles that represented full virions rather than expanded uncoating intermediates (e.g., the A(ideal)-particle). The following procedures were employed to reconstruct each of the six cryo-EM maps in this work using the program jspr39. These were full-native (dataset virus-alone), emptied (dataset virus-alone), full-d6p, full-LMWH, emptied-LMWH, and full-6’SLN. From these images, particle images were divided into two halves. For each half, particle images (5.20 Å pixel^−1) were used to generate multiple initial models by assigning random orientations to each particle. A suitable initial model was selected and used as an initial 3D reference for the refinement of particle orientations and centers. This procedure was repeated for particle images with a pixel size of 2.60 Å pixel^−1 and subsequently to those with a pixel size of 1.30 Å pixel^−1. To improve the resolution of the reconstruction, parameters for particle orientations, centers, defocus, astigmatism, scale, beam tilt, and anisotropic magnification distortion30,41 were then included in the refinement process. The resolution of the final cryo-EM map was estimated based on Fourier shell correlation (FSC) between two independently calculated half maps that were masked with a soft mask using an FSC cutoff of 0.14342,43. Maps were sharpened using a negative B-factor and low pass filtered using an FSC based filter42. In this process, the effect of modulation transfer function of the detector on the map was also corrected.

Model building and refinement. The following procedures were applied to each of the six structures presented in this work. A predicted atomic model of strain 947 was generated based on amino acid sequence comparison of strains 947 and US/MO14-18947 using SWISS-MODEL44. This model was refined based on the six structures presented in this work. A predicted atomic model of strain 947 was generated based on amino acid sequence comparison of strains 947 and US/MO14-18947 using SWISS-MODEL44. This model was re

References
1. Baggen, J., Thibault, H. J., Stratging, J. R. P. M. & Van Kuppeveld, F. J. M. The life cycle of non-polio enteroviruses and how to target it. Nat. Rev. Microbiol. 16, 368–381 (2018).
2. Strauss, M., Levy, H. C., Bostina, M., Filman, D. J. & Hogle, J. M. RNA transfer from poliovirus 135S particles across membranes is mediated by long unimolecular connectors. J. Virol. 87, 3903–3914 (2013).
3. Panjwani, A. et al. Capsid protein VP4 of human rhinovirus induces membrane permeability by the formation of a size-selective multicore pore. PLoS Pathog. 10, 1–12 (2014).
4. Hussain, K. M., Leong, K. L. J., Ng, M. M.-L. & Chu, J. J. H. The essential role of clathrin-mediated endocytosis in the infectious entry of human enterovirus 71. J. Biol. Chem. 286, 309–321 (2011).
5. Pechla, E., Kuechler, E., Blaas, D. & Fuchs, R. Uncoating of human rhinovirus type 2 from late endosomes. J. Virol. 68, 3713–3723 (1994).
6. Rosmann, M. G., He, Y. & Kuhn, R. J. Picornavirus-receptor interactions. Trends Microbiol. 10, 324–331 (2002).
7. Wang, X. et al. A sensor-adaptor mechanism for enterovirus uncoating from structures of EV71. Nat. Struct. Mol. Biol. 19, 424–429 (2012).
8. Staring, J. et al. PLAZG16 represents a switch between entry and clearance of Picoviridae. Nature 541, 412–416 (2017).
9. Elling, U. et al. A reversible haploid mouse embryonic stem cell biobank resource for functional genomics. Nature 550, 114–118 (2017).
10. Pang, X. Y. et al. Structure/function relationships of adipose phospholipase A2 type 2 from late endosomes. J. Virol. 68, 3713–3723 (1994).
11. Uyama, T. et al. The tumor suppressor gene H-Ras107 functions as a novel Ca2+–independent cytosolic phospholipase A2/1 of the thiol hydrolase type. J. Lipid Res 50, 685–693 (2009).
12. Jaworski, K. et al. AdPLA ablation increases lipolysis and prevents obesity induced by high-fat feeding or leptin deficiency. Nat. Med. 15, 159–168 (2009).
13. Messarca, K. et al. A cluster of acute flaccid paralysis and cranial nerve dysfunction temporally associated with an outbreak of enterovirus D68 in children in Colorado, USA. Lancet 385, 1662–1671 (2015).
14. Holm-Hansen, C. C., Midgley, S. E. & Fischer, T. K. Global emergence of enterovirus D68: a systematic review. Lancet Infect. Dis. 16, e64–e75 (2016).
15. Baggen, J. et al. Enterovirus D68 receptor requirements unveiled by haplotype genetics. Proc. Natl. Acad. Sci. 113, 1399–1404 (2016).
16. Liu, Y. et al. Sialic acid-dependent cell entry of human enterovirus D68. Nat. Commun. 6, 1–7 (2015).
17. Wei, W. et al. ICAM-5/teneselchalin is a functional entry receptor for enterovirus D68. Cell Host Microbe 20, 631–641 (2016).
18. Cheng, Q. et al. Atomic structures of enterovirus D68 in complex with two monoclonal antibodies define distinct mechanisms of viral neutralization. Nat. Microbiol. 4, 124–133 (2019).
19. Wang, D. et al. Andrographolide prevents EV-D68 replication by inhibiting the acidification of virus-containing endocytic vesicles. Front. Microbiol. 9, 2407 (2018).
20. Liu, Y. et al. Molecular basis for the acid initiated uncoating of human enterovirus D68. Proc. Natl Acad. Sci. USA 115, 1–63 (2018).
21. Pillary, S. et al. An essential receptor for adeno-associated virus infection. Nature 530, 108–112 (2016).
22. Baggen, J. et al. Role of enhanced receptor engagement in the evolution of a pandemic acute hemorrhagic conjunctivitis virus. Proc. Natl Acad. Sci. USA 115, 397–402 (2018).
23. Dhindwal, S., Avila, B., Feng, S. & Khayat, R. Porcine circovirus 2 uses a multitude of weak binding sites to interact with heparan sulfate, and the interactions do not follow the symmetry of the capsid. J. Virol. 93, e02222–18 (2019).
24. Levy, H. C., Bostina, M., Filman, D. J. & Hogle, J. M. Catching a virus in the act of RNA release: a novel poliovirus uncoating intermediate characterized by cryo-electron microscopy. J. Virol. 84, 4426–4441 (2010).
25. Bostina, M., Levy, H., Filman, D. J. & Hogle, J. M. Poliovirus RNA is released from the capsid near a twofold symmetry axis. J. Virol. 85, 776–783 (2011).
26. Fry, E. et al. The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. EMBO J. 18, 543–554 (1999).
27. Zhang, Y. et al. Genetic changes found in a distinct clade of Enterovirus D68 associated with paralysis during the 2014 outbreak. Virus Evol. 2, evw015 (2016).

Received: 2 August 2018 Accepted: 25 June 2019
Published online: 18 July 2019
28. Wessels, E. & Duijings, D. A proline-rich region in the coxsackievirus 3A protein is required for the protein to inhibit endoplasmic reticulum-to-Golgi transport. *J. Virol.* 79, 5163–5173 (2005).

29. Carette, J. E. et al. Haploid genetic screens in human cells identify host factors used by pathogens. *Science* 326, 1231–1235 (2009).

30. Blomen, V. A. et al. Gene essentiality and synthetic lethality in haploid human cells. *Science* 350, 1092–1096 (2015).

31. Jee, L. T. et al. Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. *Science* 340, 479–484 (2013).

32. Lanke, K. H. W. et al. GBF1, a guanine nucleotide exchange factor for Arf, is crucial for coxsackievirus B3 RNA replication. *J. Virol.* 83, 11940–11949 (2009).

33. Suloway, C. et al. Automated molecular microscopy: the new Legion system. *J. Struct. Biol.* 151, 41–60 (2005).

34. Tang, G. et al. EMA2: an extensible image processing suite for electron microscopy. *J. Struct. Biol.* 157, 38–46 (2007).

35. Zheng, S. Q. et al. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat. Methods* 14, 331–332 (2017).

36. Grant, T. & Grigorieff, N. Measuring the optimal exposure for single particle cryo-EM using a 2.6 Å reconstruction of rotavirus VP6. *Elife* 4, 1–15 (2015).

37. Rohou, A. & Grigorieff, N. CTFFIND4: fast and accurate defocus estimation from electron micrographs. *J. Struct. Biol.* 192, 216–221 (2015).

38. Scheres, S. H. W. RELION: implementation of a Bayesian approach to cryo-EM structure determination. *J. Struct. Biol.* 180, 519–530 (2012).

39. Guo, F. & Jiang, W. Single particle cryo-electron microscopy and 3-D reconstruction of viruses. *Methods Mol. Biol.* 1117, 401–443 (2014).

40. Grant, T. & Grigorieff, N. Automatic estimation and correction of anisotropic magnification distortion in electron microscopes. *J. Struct. Biol.* 192, 204–208 (2015).

41. Yu, G. et al. An algorithm for estimation and correction of anisotropic magnification distortion of cryo-EM images without need of pre-calibration. *J. Struct. Biol.* 195, 207–215 (2016).

42. Mindell, J. A. & Grigorieff, N. Accurate determination of local defocus and specimen tilt in electron microscopy. *J. Struct. Biol.* 142, 334–347 (2003).

43. Scheres, S. H. W. & Chen, S. Prevention of overfitting in cryo-EM structure determination. *Nat. Methods* 9, 853–854 (2012).

44. Biasini, M. et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* 42, 252–258 (2014).

45. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. Sect. D. Biol. Crystallogr.* 66, 213–221 (2010).

46. Afonine, P. V. et al. Real-space refinement in PHENIX for cryo-EM and crystallography. *Acta Crystallogr. Sect. D. Biol. Crystallogr.* 74, 531–544 (2018).

47. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. Sect. D. Biol. Crystallogr.* 66, 486–501 (2010).

48. Murshudov, G. N. et al. REFMACS for the refinement of macromolecular crystal structures. *Acta Crystallogr. Sect. D. Biol. Crystallogr.* 67, 355–367 (2011).

49. Chen, V. B. et al. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. Sect. D. Biol. Crystallogr.* 66, 12–21 (2010).

50. Petersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25, 1605–1612 (2004).

51. Xiao, C. & Rossmann, M. G. Interpretation of electron density with stereographic roadmap projections. *J. Struct. Biol.* 158, 182–187 (2007).

**Acknowledgements**

Funding was provided by the Netherlands Organization for Scientific Research grant NWO-VICI-91812628 (to F.J.M.v.K.) and by National Institute of Health of the United States, NIAID grant AI011219 (to M.G.R.) We thank Jacqueline Staring for assistance with the genetic screen, Elmer Stickel and Vincent Blomen for assistance with data analysis, and prof. dr. Thijn Brummelkamp for his guidance. We thank Prof. Dr. Geert-Jan Boons for his valuable advice regarding the choice of sGAG analogs. We are grateful to Thomas Klöse, Yingyuan Sun, Wen Jiang, and Valerie Bowman for help with cryo-EM analysis.

**Author contributions**

J.B. was involved in performing the genetic screen, J.B., H.L., A.L.W.v.V., M.W. and R.W.R. performed infectivity assays. Y.L. performed cryo-EM experiments and data processing. A.L.W.v.V. and J.W.D.B. prepared mutant viruses. P.O. and A.M. analyzed viral sequences in patient material. J.B., H.J.T. and F.J.M.v.K designed the project. M.G.R and F.J.M.v.K supervised and supported the project. J.B., Y.L., H.J.T and F.J.M.v.K. wrote the paper.

**Additional information**

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11256-z.

**Competing interests:** The authors declare no competing interests.

**Reprints and permission** information is available online at http://npg.nature.com/reprintsandPermissions/

**Peer review information:** *Nature Communications* thanks Satoshi Koike and other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.