Correction

MICROBIOLOGY

Correction for "Enterovirus D68 receptor requirements unveiled by haploid genetics," by Jim Baggen, Hendrik Jan Thibaut, Jacqueline Staring, Lucas T. Jae, Yue Liu, Hongbo Guo, Jasper J. Slager, Jost W. de Bruin, Arno L. W. van Vliet, Vincent A. Blomen, Pieter Overduin, Ju Sheng, Cornelis A. M. de Haan(Xander), Erik de Vries, Adam Meijer, Michael G. Rossmann, Thijn R. Brummelkamp, and Frank J. M. van Kuppeveld, which appeared in issue 5, February 2, 2016, of *Proc Natl Acad Sci USA* (113:1399–1404; first published January 19, 2016; 10.1073/pnas.1524498113).

The authors note that the author name Cornelis A. M. de Haan(Xander) should instead appear as Cornelis A. M. de Haan. The corrected author line appears below. The online version has been corrected.

Jim Baggen, Hendrik Jan Thibaut, Jacqueline Staring, Lucas T. Jae, Yue Liu, Hongbo Guo, Jasper J. Slager, Jost W. de Bruin, Arno L. W. van Vliet, Vincent A. Blomen, Pieter Overduin, Ju Sheng, Cornelis A. M. de Haan, Erik de Vries, Adam Meijer, Michael G. Rossmann, Thijn R. Brummelkamp, and Frank J. M. van Kuppeveld

www.pnas.org/cgi/doi/10.1073/pnas.1602316113

Enterovirus D68 receptor requirements unveiled by haploid genetics

Jim Baggen[a,1], Hendrik Jan Thibaut[a,1], Jacqueline Staring[b], Lucas T. Jae[b], Yue Liu[c], Hongbo Guo[a], Jasper J. Slager[a], Jost W. de Bruin[a], Arno L. W. van Vliet[a], Vincent A. Blomen[b], Pieter Overduin[d], Ju Sheng[c], Cornelis A. M. de Haan[a], Erik de Vries[a], Adam Meijer[d], Michael G. Rossmann[c,2], Thijn R. Brummelkamp[b,e], and Frank J. M. van Kuppeveld[a,2]

[a]Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3584 CL, Utrecht, The Netherlands; [b]Biochemistry Division, Netherlands Cancer Institute, 1006 BE, Amsterdam, The Netherlands; [c]Department of Biological Sciences, Purdue University, West Lafayette, IN 47907; [d]Virology Division, Centre for Infectious Diseases Research, Diagnostics and Screening, National Institute for Public Health and the Environment, 3720 BA, Bilthoven, The Netherlands; and [e]Cancer Genomics Centre, 3584 CG, Utrecht, The Netherlands

Contributed by Michael G. Rossmann, December 14, 2015 (sent for review October 8, 2015; reviewed by David J. Rowlands and Thilo Stehle)

Enterovirus D68 (EV-D68) is an emerging pathogen that can cause severe respiratory disease and is associated with cases of paralysis, especially among children. Heretofore, information on host factor requirements for EV-D68 infection is scarce. Haploid genetic screening is a powerful tool to reveal factors involved in the entry of pathogens. We performed a genome-wide haploid screen with the EV-D68 prototype Fermon strain to obtain a comprehensive overview of cellular factors supporting EV-D68 infection. We identified and confirmed several genes involved in sialic acid (Sia) biosynthesis, transport, and conjugation to be essential for infection. Moreover, by using knockout cell lines and gene reconstitution, we showed that both α2,6- and α2,3-linked Sia can be used as functional cellular EV-D68 receptors. Importantly, the screen did not reveal a specific protein receptor, suggesting that EV-D68 can use multiple redundant sialylated receptors. Upon testing recent clinical strains, we identified strains that showed a similar Sia dependency, whereas others could infect cells lacking surface Sia, indicating they can use an alternative, nonsialylated receptor. Nevertheless, these Sia-independent strains were still able to bind Sia on human erythrocytes, raising the possibility that these viruses can use multiple receptors. Sequence comparison of Sia-dependent and Sia-independent EV-D68 strains showed that many changes occurred near the canyon that might allow alternative receptor binding. Collectively, our findings provide insights into the identity of the EV-D68 receptor and suggest the possible existence of Sia-independent viruses, which are essential for understanding tropism and disease.

enterovirus D68 | haploid genetic screen | receptor | sialic acid

The genus *Enterovirus* of the family *Picornaviridae* contains many important pathogens for humans and animals. This genus consists of 12 species: four human enterovirus species (*EV-A*, *-B*, *-C*, and *-D*), five animal enterovirus species, and three human rhinovirus species. The best known human enterovirus is poliovirus (*EV-C*), the cause of poliomyelitis and acute flaccid paralysis. Other well-known enteroviruses are the coxsackieviruses (*EV-B* and *EV-C*)—which are the main cause of viral meningitis, conjunctivitis, myocarditis, and herpangina—and enterovirus A71, which causes hand-foot-and-mouth disease and is also associated with severe neurological disease, causing serious public health concerns in Southeast Asia (1).

Another emerging enterovirus that causes growing public health problems is enterovirus D68 (EV-D68, a member of the species EV-D). Unlike most enteroviruses, which are acid-resistant and multiply in the human gastrointestinal tract, EV-D68 is an acid-sensitive enterovirus (2) that replicates in the respiratory tract. EV-D68 was first isolated from children with respiratory infections in California in 1962 (3). It was long considered a rare pathogen, but the frequency of detecting EV-D68 during outbreaks of respiratory disease has increased (4, 5) and over the past decades, three clades of EV-D68 (A, B, and C) have emerged and spread worldwide (6, 7). EV-D68 infections mostly cause mild respiratory disease but can also result in severe bronchiolitis or pneumonia, especially among children (4, 5). In 2014, a nationwide EV-D68 outbreak in the United States was associated with severe respiratory disease and a cluster of acute flaccid myelitis and cranial nerve dysfunction in children, implicating EV-D68 as an emerging public health threat (8, 9).

Enteroviruses are small, nonenveloped viruses that contain a single-stranded RNA genome of positive polarity. To initiate infection, enteroviruses bind to specific receptors on host cells. To date, most known enterovirus receptors are cell surface proteins, many of which belong to the Ig superfamily or the integrin receptor family (10). A majority of these receptors bind to the "canyon," a depression on the virion surface, thereby destabilizing virions and initiating uncoating (11). In EV-D68, the canyon is unusually shallow and narrow, possibly excluding use of large protein receptors (12). Both sensitivity of EV-D68 infection to neuraminidase (NA) treatment and hemagglutination assays point to the use of Sia as the receptor (13, 14). However, beside the role of a terminal Sia residue on the receptor, little is known about the type(s) of Sia that can be used by

Significance

Enterovirus D68 (EV-D68) is an emerging pathogen that recently caused a large outbreak of severe respiratory disease in the United States and is associated with cases of paralysis. Little is known about EV-D68 host factor requirements. Here, using a genome-wide knockout approach, we identified several genes in sialic acid (Sia) biology as being essential for infection. We also showed that not only α2,6-linked Sia, which mainly occurs in the upper respiratory tract, but also α2,3-linked Sia, which mainly occurs in the lower respiratory tract, can serve as the receptor. Moreover, we identified recent EV-D68 isolates that can use an alternative, nonsialylated receptor. Our findings are essential to understand tropism and pathogenesis of EV-D68 as well as the potential of using Sia-targeting inhibitors to treat EV-D68 infections.

Author contributions: J.B., H.J.T., M.G.R., T.R.B., and F.J.M.v.K. designed research; J.B., H.J.T., J. Staring, L.T.J., H.G., J.J.S., J.W.d.B., A.L.W.v.V., and P.O. performed research; J.B., H.J.T., L.T.J., Y.L., V.A.B., J. Sheng, C.A.M.d.H., E.d.V., and A.M. analyzed data; and J.B., H.J.T., M.G.R., T.R.B., and F.J.M.v.K. wrote the paper.

Reviewers: D.J.R., University of Leeds; and T.S., University of Tuebingen.

The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KT231897–KT231908).

[1]J.B. and H.J.T. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: mr@purdue.edu or F.J.M.vanKuppeveld@uu.nl.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1524498113/-/DCSupplemental.

EV-D68 to infect cells, the composition of the underlying glycan, and whether specific sialylated proteins or glycolipids are required for infection.

Genome-wide genetic screening in human haploid cells is a powerful tool to reveal host factors involved in entry of various pathogens, including viruses (15, 16). In this study, we performed a haploid screen and demonstrate that genes involved in synthesis of sialylated glycans are essential for EV-D68 infection. Furthermore, we show that EV-D68 is able to use α2,6-linked as well as α2,3-linked Sia as a cellular receptor, and we provide the first insights into the composition of the underlying sugar backbone. Finally, we report the identification of recent EV-D68 isolates that can infect Sia-deficient cells, indicating that these strains can use an alternative receptor.

Results

Multiple Genes Involved in Sia Biology Determine Susceptibility of Cells to EV-D68 Infection. We performed a haploid genetic screen (17, 18) by infecting mutagenized human HAP1 cells with the EV-D68 prototype strain Fermon CA62-1. The screen identified nine genes involved in Sia biology (Fig. 1 *A* and *B* and Fig. S1), seven of which were in the top 10. Among these nine hits are genes involved in the biosynthesis [UDP-GlcNAc-2-epimerase/ManAc kinase (*GNE*) and N-acetylneuraminic acid synthase (*NANS*)] and activation [cytidine monophosphate N-acetylneuraminic acid synthetase (*CMAS*)] of N-acetylneuraminic acid, the predominant form of Sia in humans. Other hits include transporters that transfer the activated sugars CMP-Sia and UDP-galactose [solute carrier family 35 member A1 and A2 (*SLC35A1* and *SLC35A2*)] from the cytosol to the Golgi apparatus and four glycosyltransferases responsible for conjugation of N-acetylglucosamine (GlcNAc) to mannose residues in N-linked glycans [mannoside acetylglucosaminyltransferase 5 (*MGAT5*)], galactose [beta-1,4-galactosyltransferase 1 (*B4GALT1*)], and Sia, either via α2,3 linkage [ST3 beta-galactoside alpha-2,3-sialyltransferase 4 (*ST3GAL4*)] or α2,6 linkage [ST6 beta-galactosamide alpha-2,6-sialyltranferase 1 (*ST6GAL1*)]. Together, these data provide insights into the identity and composition of the EV-D68 receptor, pointing to an important role of α2,6- and α2,3-linked Sia on *N*-linked glycans in infection.

To confirm the results of the genetic screen, we used mutant cell lines lacking surface expression of Sia (*SLC35A1*^{KO} and *CMAS*^{KO}) or having a defect in formation of α2,3- and/or α2,6-linked Sia (*ST3GAL4*^{KO}, *ST6GAL1*^{KO}, and *ST3GAL4*/*ST6GAL1*^{DKO}). The integrity of these mutant cell lines was confirmed by genetic analysis (Fig. S2 *A* and *B*), lectin stainings (Fig. S2*C*), and infection with Sia-dependent and -independent control viruses. Analysis of the number of infected cells showed that *SLC35A1*^{KO} and *CMAS*^{KO} cells were highly resistant to influenza A virus (IAV), whereas *ST3GAL4*/*ST6GAL1*^{DKO} cells were partially resistant (Fig. 2*A*), consistent with the broad Sia specificity of IAV (19). In contrast, coxsackievirus-B3, which does not require Sia, could infect all cell lines (Fig. 2 *A* and *B*), whereas equine rhinitis A virus, a picornavirus that requires α2,3-linked Sia (20), could infect *ST6GAL1*^{KO} but not *ST3GAL4*^{KO} cells (Fig. 2*C*). Upon characterizing EV-D68 Fermon, we observed that infection was inhibited by NA treatment and almost completely blocked in *CMAS*^{KO}, *SLC35A1*^{KO}, and *ST3GAL4*/*ST6GAL1*^{DKO} cells, both at high (Fig. 2*A* and Fig. S2*D*) and low multiplicity of infection (moi) (Fig. 2*B*). Likewise, little, if any, production of progeny virus was observed in these mutant cell lines (Fig. 2*C*).

EV-D68 Can Use Both α2,6- and α2,3-Linked Sia to Infect Cells. Specificity for α2,3- or α2,6-linked Sia can greatly affect tissue tropism of respiratory viruses, as the Sia abundance varies between the upper (mainly α2,6-linked) and lower (mainly α2,3-linked) respiratory tract (21, 22). Identification of *ST3GAL4* and *ST6GAL1* suggested that both α2,6- and α2,3-linked Sia are used for infection. Indeed, *ST6GAL1*^{KO} cells were less susceptible to EV-D68, whereas *ST3GAL4*^{KO} cells were equally susceptible as wild-type cells (Fig. 2 *A*–*C*), suggesting a preference of EV-D68 for α2,6-linked Sia. However, EV-D68 can also use α2,3-linked Sia as a receptor, as shown by the observation that *ST3GAL4*/*ST6GAL1*^{DKO} cells were more resistant to infection than *ST6GAL1*^{KO} cells (Fig. 2 *A*–*C*). Consistently, *ST3GAL4*/*ST6GAL1*^{DKO} cells could be rendered more susceptible to EV-D68 by transfection of plasmid containing *ST3GAL4* cDNA (and also *ST6GAL1*), but the effect was small due

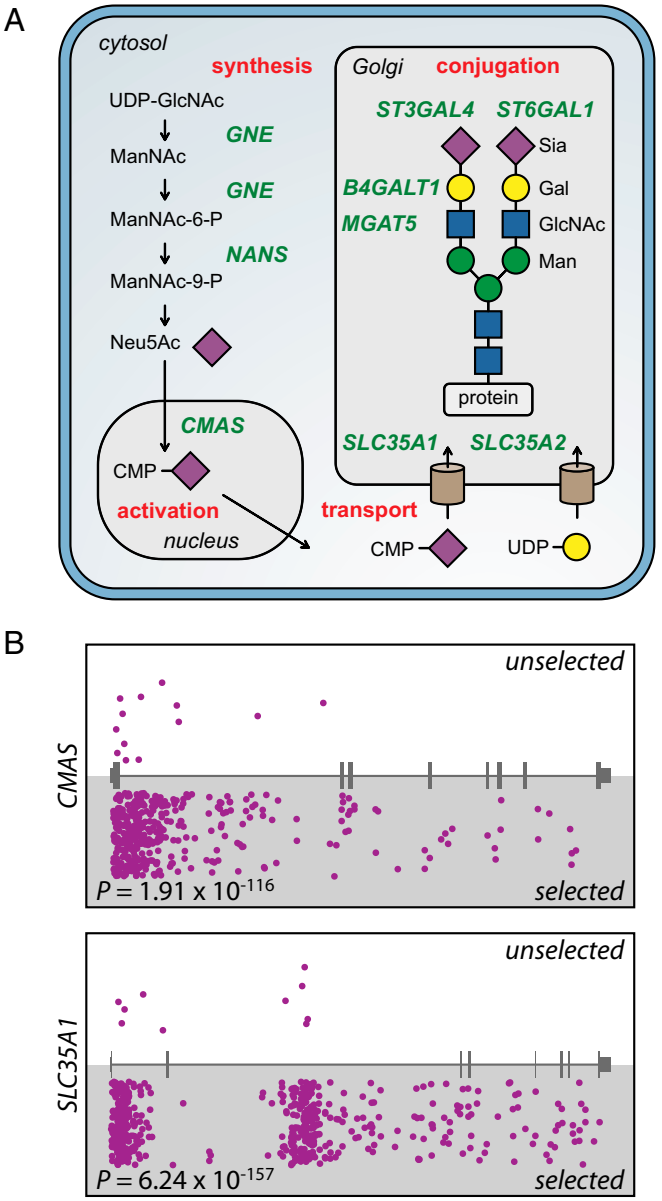

Fig. 1. A haploid genetic screen for EV-D68 identifies genes involved in synthesis of sialylated glycans. (*A*) An overview of the different steps in synthesis of Sia and sialylated *N*-linked glycans. Significant hits related to Sia biology are shown in green. Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Sia, sialic acid. (*B*) RefSeq gene structures of *CMAS* and *SLC35A1* are shown with the transcriptional orientation pointing from left to right. Gene-trap insertions predicted to disrupt gene function (intronic insertions in sense orientation and insertions mapping to exons) are illustrated for HAP1 cells selected with EV-D68 (selected) and a cell population of similar complexity that had not been selected with EV-D68 (unselected). The FDR-corrected *P* values for the enrichment of disruptive gene-trap mutations in the EV-D68–selected population are indicated (*Materials and Methods*). For insertion plots of the other hits, see Fig. S1.

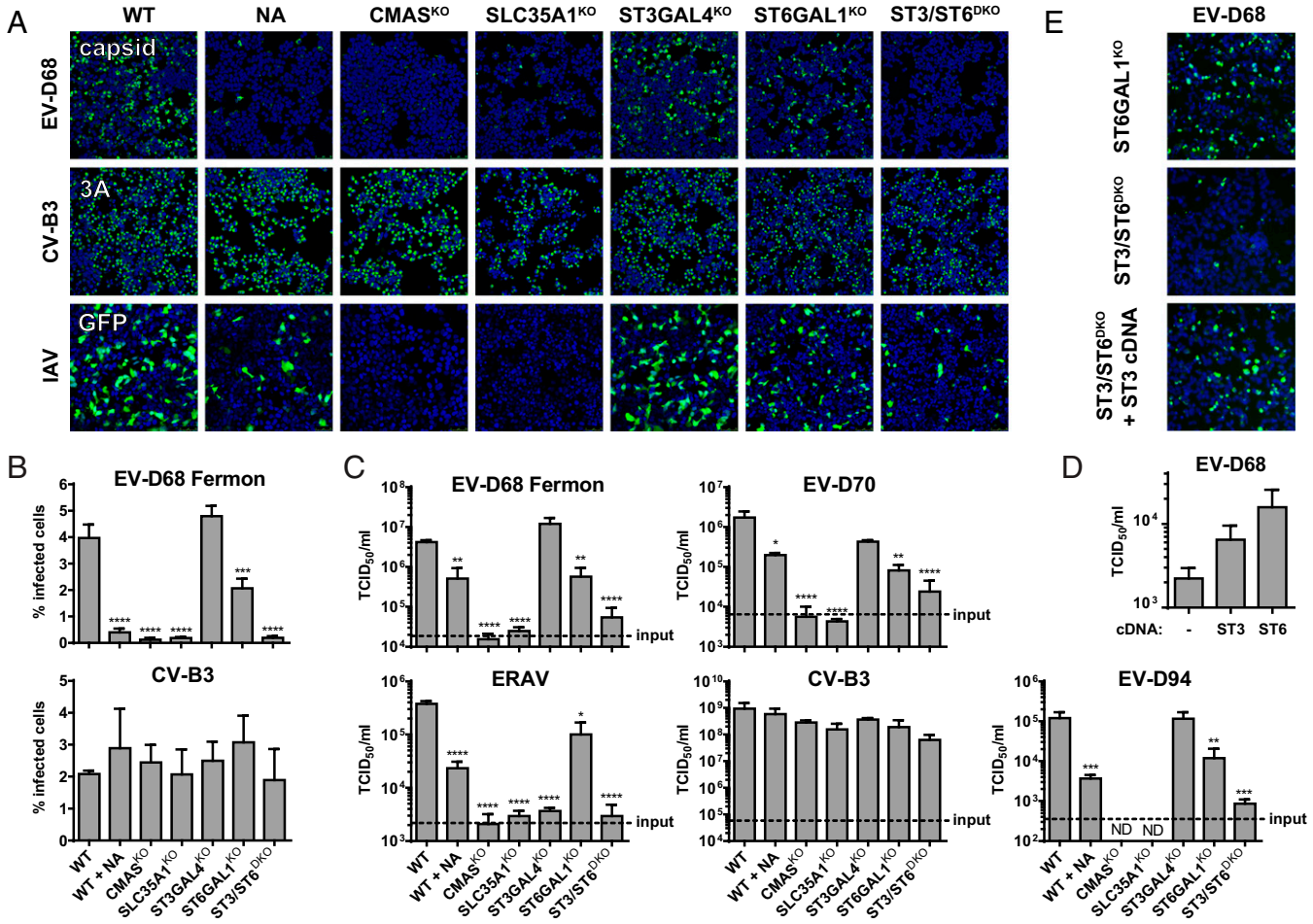

Fig. 2. Both α2,3- and α2,6-linked Sia can support EV-D68 infection. (*A*) HAP1 cells treated with NA or HAP1 clones having defects in Sia synthesis were challenged with EV-D68 (Fermon), CV-B3, or IAV-GFP at an moi of 10. Infected cells were visualized by immunostaining and fluorescence microscopy. (*B*) Cells were infected at an moi of 0.1, and the percentage of infected cells was determined by immunostaining and counting. The mean ± SEM of four biological replicates is shown. (*C*) Yields of infectious virus after a single cycle of virus production. ERAV, equine rhinitis A virus; EV-D70, enterovirus-D70; EV-D94, enterovirus-D94. The mean ± SEM of three biological replicates is shown. In panels *B* and *C*, *P* values compared with WT were calculated by one-way ANOVA of log-transformed data with Bonferroni correction. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. (*D*) *ST3GAL4/ST6GAL1*^{DKO} cells transfected with plasmids containing *ST3GAL4* or *ST6GAL1* cDNA were exposed to EV-D68, and yields of infectious virus were measured after a single replication cycle. (*E*) *ST6GAL1*^{KO} or *ST3GAL4/ST6GAL1*^{DKO} cells (ST3/ST6^{DKO}) transduced with retrovirus expressing *ST3GAL4* cDNA were exposed to EV-D68 and stained with antibody against the viral capsid.

to the low transfection efficiency in HAP1 cells (Fig. 2*D*). Upon retroviral transduction with *ST3GAL4* cDNA, however, high infection efficiency was observed (Fig. 2*E*).

Identification of *ST3GAL4* in the genetic screen is likely due to heterogeneous expression of α2,3- and α2,6-linked Sia, which has been described in human airway epithelial cultures but also occurs in cultured cells, including HAP1 (Fig. S2*E*). Hence, knockout of *ST3GAL4* in cells already expressing α2,6-linked Sia at reduced levels likely conferred resistance to infection. In summary, our data show that both α2,6- and α2,3-linked Sia can be used for infection by EV-D68.

Other EV-D Members Display a Similar Sia Preference Profile. We also investigated the Sia dependency of EV-D70 and EV-D94, two other members of the EV-D species. EV-D70 causes outbreaks of hemorrhagic conjunctivitis, which are often associated with neurological disorders (23). EV-D94 has been associated with acute flaccid paralysis (24), but information on the clinical relevance of this virus is scarce. EV-D70 was reported to be NA-sensitive (25), whereas any role of Sia in EV-D94 infection is unknown. Like EV-D68 Fermon, EV-D70 and EV-D94 could not replicate in *CMAS*^{KO} and *SLC35A1*^{KO} cells. Also, *ST3GAL4/ST6GAL1*^{DKO} cells were less susceptible to infection than *ST6GAL1*^{KO} cells (although differences for EV-D70 were less pronounced), suggesting that EV-D70 and EV-D94 can use both α2,6- and α2,3-linked Sia as the receptor (Fig. 2*C*). Altogether, these data suggest conserved Sia use between EV-D members.

Identification of EV-D68 Strains That Can Infect Cells in a Sia-Independent Manner. EV-D68 Fermon was isolated more than 50 y ago and genetically differs from currently circulating strains. Therefore, we investigated the Sia dependence of six recent EV-D68 strains—belonging to the three different EV-D68 clades—isolated from patients with respiratory infections in 2009 and 2010 in the Netherlands (5). To minimize the emergence of genetic variants, these isolates were subjected to a limited number of passages in cell culture before they were analyzed for their ability to infect different HAP1 cell lines. Like EV-D68 Fermon, three clinical strains (670, 2042, and 2284) did not cause cytopathic effect (CPE) in Sia-deficient cells (Fig. 3*A*) and were sensitive to NA treatment, as shown by analysis of virus production in a single cycle (Fig. 3*B*) or multiple cycles of replication (Fig. S3 *A* and *B*). These strains, like Fermon, exhibited some virus production in *ST6GAL1*^{KO} cells, despite the lack of

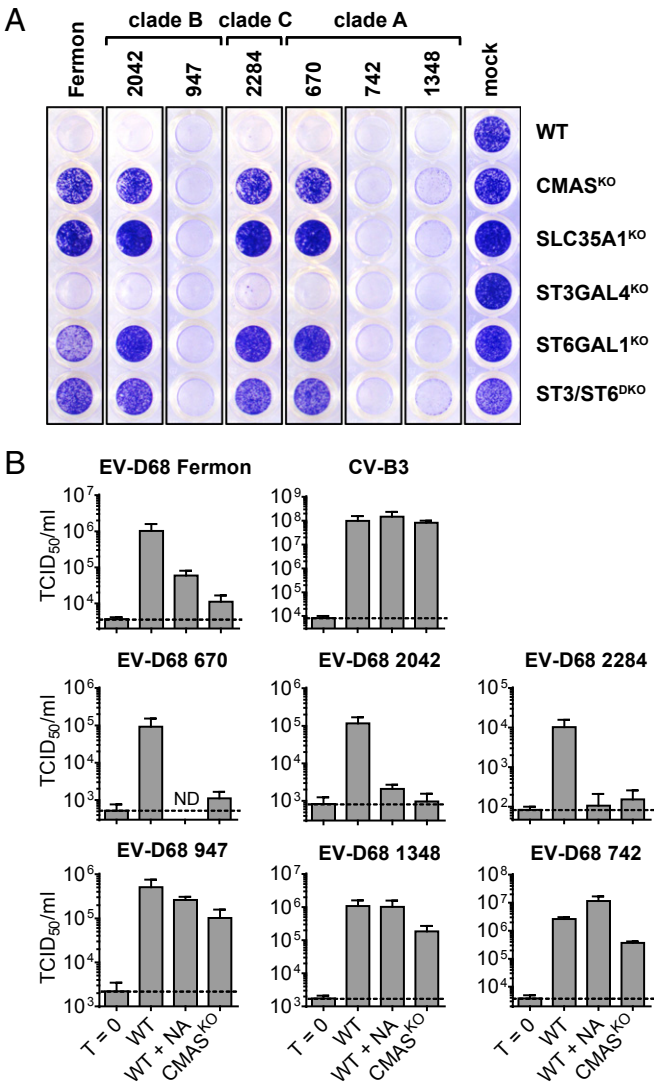

Fig. 3. Identification of EV-D68 strains that can infect cells independently of Sia. (*A*) HAP1 clones were infected with different EV-D68 strains and stained with crystal violet. (*B*) HAP1 cells treated with NA or *CMAS*^{KO} cells were infected with different EV-D68 strains, and yields of infectious virus were measured after a single cycle of replication. Virus input levels (T = 0) are indicated by a dashed line. ND, not detectable. The mean ± SEM of three biological replicates is shown.

CPE (Fig. S3*A*). Strikingly, the other three strains (947, 1348, and 742) were able to replicate in Sia-deficient and NA-treated HAP1 cells (Fig. 3 *A* and *B*). Sia independence of these strains was not specific for HAP1 cells, as these viruses also efficiently infected NA-treated A549 and HeLa-R19 cells (Fig. S3*C*). In summary, these data demonstrate that several recent EV-D68 strains strongly depend on Sia, whereas other strains can infect cells in a Sia-independent manner, pointing toward the use of a nonsialylated receptor.

Sia-Independent EV-D68 Strains Retain Sia-Binding Capacity. A recent study showed that, in vitro, Sia-containing trisaccharides can bind to EV-D68, where the floor of the canyon would be in the major group rhinoviruses or polioviruses (26) (Fig. 4). To gain insight into the residues that allow Sia-independent infection and their location with respect to the Sia-binding site, we sequenced the capsid regions of the different EV-D68 strains. Amino acid sequence comparison showed that the genetically related Sia-dependent (670) and -independent (742 and 1348) strains in clade A differed at 10 positions, whereas the Sia-dependent (2042) and -independent (947) strains in clade B differed at seven positions (Fig. 4 and Tables S1 and S2). Although residues that differed between Sia-dependent and -independent strains in clade A show little overlap with those that were altered in clade B, some of the changed residues on the viral surface are near the Sia-binding site (26) (Fig. 4).

To investigate whether the amino acid substitutions that established an alternative receptor-binding site might have affected the Sia-binding capacity of these strains, we performed hemagglutination experiments with blood from nine different human donors. All EV-D68 isolates agglutinated human erythrocytes, although two Sia-independent strains (1348 and 742) agglutinated erythrocytes from only one donor (Table 1). Pretreatment of erythrocytes with NA prevented hemagglutination by EV-D68 strains but not by echovirus-7, which agglutinates erythrocytes by binding to its protein receptor, decay-accelerating factor (27). Remarkably, blood from three donors was not agglutinated by any of the EV-D68 strains, whereas hemagglutination titers of IAV were similar for all donors, indicating equal Sia expression levels. This variability suggests that EV-D68 does not merely bind any sialylated glycan but has a preference for specific sialylated glycan structures that are differentially expressed between individuals. No clear correlation between EV-D68 hemagglutination and ABO blood groups was observed. In summary, these data indicate that Sia-independent strains have retained their Sia-binding capacity, albeit two strains (742 and 1348) seem to have a reduced affinity for Sia.

Discussion

In this study, we provided important insights into the identity/nature of the EV-D68 receptor. Using a genome-wide haploid screen, we identified genes involved in biosynthesis (*GNE* and *NANS*), activation (*CMAS*), transport (*SLC35A1*), and conjugation of Sia to glycans (*ST3GAL4* and *ST6GAL1*) as factors required for EV-D68 infection. Using knockout cell lines and gene reconstitution, we have shown that EV-D68 can use both α2,6- and α2,3-linked Sia as a receptor to infect cells. This finding extends recent observations that both α2,6- and α2,3-linked Sia-containing trisaccharides can bind to the EV-D68 capsid and initiate virion uncoating in vitro (26). The observation that EV-D68 can use not only α2,6-linked Sia as a receptor but also α2,3-linked Sia, which resides mainly in the lower respiratory tract, may provide an explanation for its ability to cause severe lower respiratory tract infections.

Importantly, the screen did not identify a specific protein receptor, suggesting that EV-D68 can use multiple redundant receptors, given these are glycosylated with a suitable sialylated glycan. Our screen also provided insights into the preference of EV-D68 for specific sialylated glycans. Identification of glycosyltransferases responsible for conjugation of galactose (*B4GALT1*) and GlcNAc (*MGAT5*) pointed to the importance of Sia–galactose–GlcNAc chains, consistent with the substrate specificity of ST3GAL4 and ST6GAL1. Furthermore, the identification of MGAT5, which forms GlcNAc–β1,6-Man linkages, suggested that EV-D68 specifically recognizes *N*-linked glycans containing a β1,6-linked antenna. It should be noted that Sia–galactose–GlcNAc, although mainly expressed on *N*-linked glycans, also occurs on O-linked glycans and glycolipids and that we observed that HEK293S cells, which lack complex *N*-linked glycans (19), are susceptible to EV-D68. Further proof that EV-D68 does not merely bind any sialylated glycan but has a preference for specific glycans stems from our observation that erythrocytes of several donors could be agglutinated by IAV but not by EV-D68. More research is required to explore the glycan spectrum that can be bound by EV-D68.

Upon characterizing recent EV-D68 isolates, we identified strains that are able to infect Sia-deficient cells, implying that these viruses can use an alternative entry receptor. Genetic comparison of Sia-dependent and -independent EV-D68 strains within clades A and B revealed little overlap of residues determining Sia independence but pointed toward residues near the Sia-binding site as possible

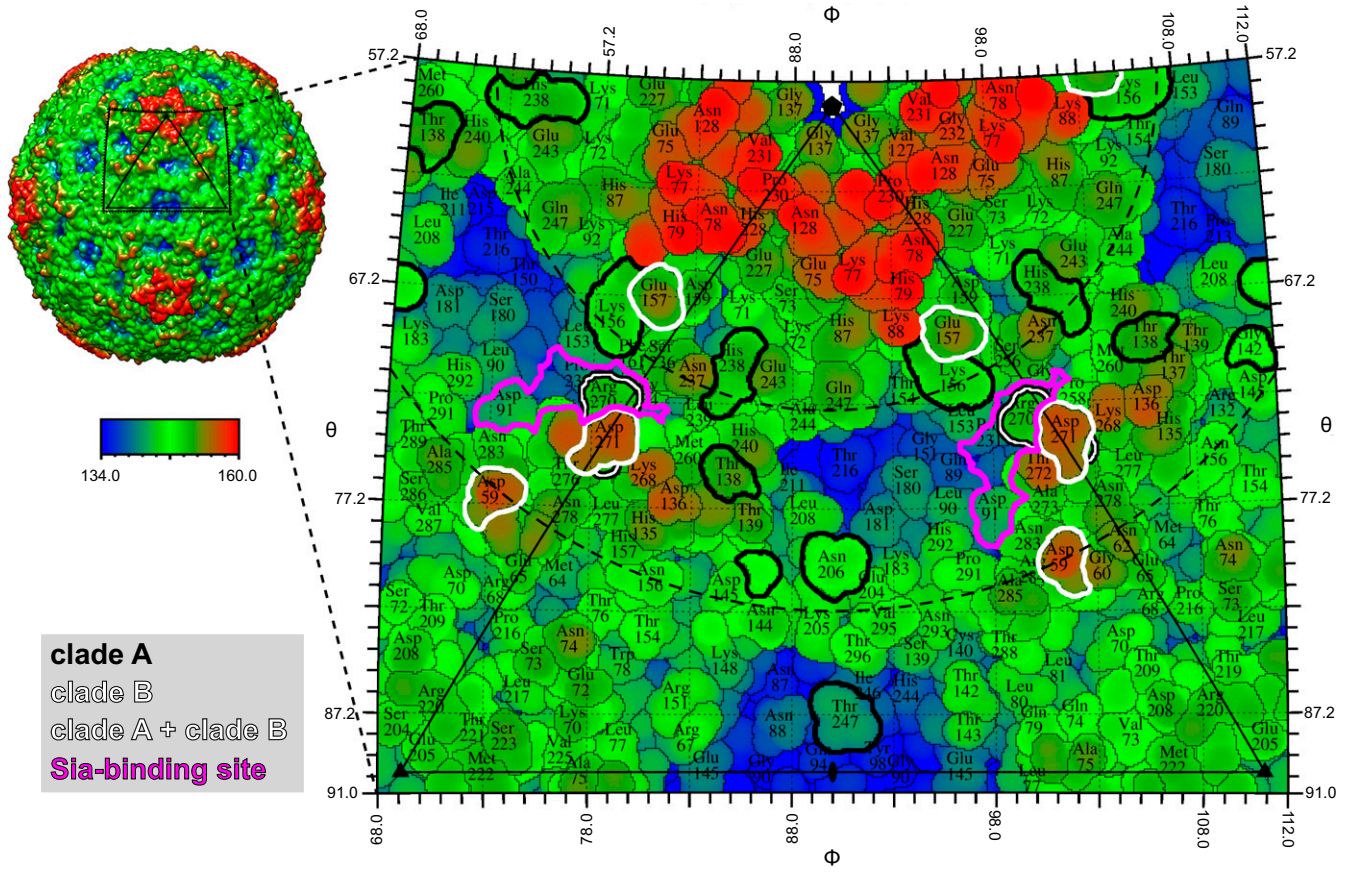

Fig. 4. A map of the EV-D68 surface residues. Surface representation of the complete EV-D68 capsid structure (12) (*Left*) and a close-up showing the Sia-binding site within the canyon (*Right*). The black triangle shows an icosahedral asymmetric unit of the EV-D68 Fermon structure. Residues are colored by radial distance (Å) to the virus center. The black dashed lines outline the canyon in the human rhinovirus 14 structure. Variable residues determining Sia (in)dependence of strains within clade A or clade B are highlighted by black and white contours, respectively. Note that residue 270 in VP1 (black and white contour) is variable both within clade A and clade B. Amino acids were numbered according to Liu et al., 2015 (12).

determinants for Sia (in)dependence. A similar scenario was described for Sia-dependent and -independent rotavirus strains, where only a few amino acid changes in the Sia-binding site could cause a receptor switch to a nonsialylated glycan (28). It remains to be investigated whether a single amino acid substitution or a combination thereof is required for the observed Sia-independent phenotype. Better understanding of the (combinations of) residues that facilitate Sia independence could ultimately allow prediction of receptor requirement based on sequence alignments.

It has been shown in vitro that Sia binds to the EV-D68 canyon at a unique site, compared with the glycan-binding sites in other picornaviruses (Fig. S4). In EV-D68, binding of Sia induces virion destabilization and pocket factor release, which is the first step in the uncoating process (26). We identified several EV-D68 strains that can use a nonsialylated receptor while retaining a Sia-binding capacity, albeit with different affinities. This indicates that the formation of an alternative receptor-binding site does not necessarily result in loss of the Sia-binding site and points to the possible existence of a dual receptor mechanism, where either Sia or a nonsialylated receptor can trigger similar structural conformational changes. However, it is unclear whether binding of Sia to the capsid of Sia-independent viruses still results in virion destabilization and pocket factor release as described for EV-D68 Fermon. Furthermore, more research is warranted to determine whether this alternative receptor is a protein or a sugar that lacks a terminal Sia moiety.

Although it remains to be established whether Sia-independent strains circulate in the human population, the occurrence of strains that use an alternative receptor could have an impact on tissue tropism and pathogenesis of EV-D68. Also, application of the sialidase DAS-181 (Fludase) (29), an investigational drug against influenza virus that was shown to inhibit EV-D68 (30), may be ineffective against Sia-independent EV-D68 strains. Hence, detailed insight into the interactions of EV-D68 with its receptor(s) is required to understand viral pathogenesis and to develop effective antiviral treatment.

Materials and Methods

Cells and Viruses. Information on viruses and cells used in this study is described in *SI Materials and Methods*.

Table 1. Hemagglutination titers (Log_2) of EV-D68 strains on human erythrocytes from nine different donors

Donor	1	2	3	4	5	6	7	8	9	1 + NA	2 + NA
EV-D68 2042	2	2	0	2	4	0	2	0	6	0	0
EV-D68 947	3	2	0	3	4	0	2	0	6	0	0
EV-D68 2284	2	2	0	3	4	0	2	0	5	0	0
EV-D68 670	3	2	0	3	3	0	2	0	7	0	0
EV-D68 742	0	0	0	0	0	0	0	0	3	0	0
EV-D68 1348	1	0	0	0	0	0	0	0	3	0	0
IAV PR8	9	9	9	10	10	9	9	9	10	0	0
IAV WSN	8	8	8	9	8	8	7	8	8	0	0
Echovirus 7	8	7	ND	ND	ND	ND	ND	ND	ND	8	9

ND, not determined.

Haploid Genetic Screen with EV-D68. HAP1 cells were gene-trap mutagenized as described previously (31). Following expansion, 10^8 mutagenized cells were exposed to EV-D68 Fermon (moi 3). After selection, surviving cells were expanded and used for genomic DNA isolation. Insertion sites identified in cells selected with EV-D68 (yielding 414,290 unique gene-trap insertions mapped to genes) and a population of matched control cells of comparable complexity (495,679 unique gene-trap insertions mapped to genes) were aligned to the human genome not filtering for close reads (31). Subsequently, disruptive insertion sites (in sense orientation of the affected gene or mapping to exons) in significantly identified genes were compared in the two cell populations, and *P* values for enrichment were calculated using a Fisher's exact test as described previously (31). Disruptive insertion sites in virus-selected and control cells were plotted onto the RefSeq gene bodies for the following transcripts: NM_001497 (*B4GALT1*), NM_018686 (*CMAS*), NM_001128227 (*GNE*), NM_002410 (*MGAT5*), NM_018946 (*NANS*), NM_006416 (*SLC35A1*), NM_005660 (*SLC35A2*), NM_006278 (*ST3GAL4*), and NM_173216.2 (*ST6GAL1*).

Generation of Knockout Cells. *ST3GAL4*^{KO} and *SLC35A1*^{KO} HAP1 cells have been described (31). *CMAS*^{KO} cells were obtained from Haplogen GmbH. The CRISPR-Cas9 system was used to generate *ST6GAL1*^{KO} cells. The entire *ST6GAL1* locus was excised (Fig. S2*A*), and subclones were analyzed by genotyping (Table S3). *ST3GAL4*/*ST6GAL1*^{DKO} cells were obtained by deleting an exonic region in *ST3GAL4* from *ST6GAL1*^{KO} cells using CRISPR-Cas9.

Infectivity Assays. Cells were infected with virus for 1 h. After incubation for the indicated period, virus titers were determined by end-point dilution. Crystal violet staining was performed at 3 d postinfection. Where indicated, cells were pretreated with NA from *Clostridium perfringens* (NEB) or from *Arthrobacter ureafaciens* (Roche) in serum-free medium for 30 min.

Immunofluorescence Assays. Paraformaldehyde-fixed cells were stained using rabbit anti-capsid serum against EV-D68 Fermon (produced in house; 1:1,000) or a mouse monoclonal antibody against CV-B3 protein 3A (1:100) (32). For characterization with lectins, cells were stained with fluorescein-labeled *Sambucus nigra* lectin (Vector Laboratories; 1:1,000) and biotinylated *Maackia amurensis* lectin I (Vector Laboratories; 1:500). Cells were examined by confocal microscopy (Leica SPE-II) or standard fluorescence microscopy (EVOS FL cell imaging system). The number of nuclei was quantified using ImageJ, and the number of infected cells was quantified visually.

Isolation and Sequencing of EV-D68 Strains from Clinical Specimen. Monolayers of tertiary monkey kidney cells (tMKs) or human rhabdomyosarcoma (RD) cells were incubated with 250 μL EV-D68–positive clinical material (mixed nose and throat swabs) derived from patients with influenza-like illness or acute respiratory infection (5) and incubated at 34 °C until CPE was observed. EV-D68 strains 4311000670 (clade A; further referred to as 670) and 4311000742 (clade A; 742) were isolated on tMK cells, whereas strains 4310900947 (clade B; 947), 4310901348 (clade A; 1348), 4310902042 (clade B; 2042), and 4310902284 (clade C; 2284) were isolated on RD cells. Viruses were harvested by one freeze-thawing cycle at –80 °C and clarified by centrifugation. Subsequent passages of virus were done on tMK or RD cell monolayers. The full genome of the virus isolates was sequenced from passage 4 for strains 670 and 742 and from passage 2 of the remaining four strains. All infection experiments described in this study were performed with viruses that had undergone one or two more rounds of passage on RD cells. From these viruses, the 5′UTR and capsid region was sequenced. The latter sequences were used for amino acid comparisons shown in Tables S1 and S2. Detailed information on virus passages, sequences, and GenBank accession numbers is described in *SI Materials and Methods*. As required by Dutch legislation, surveillance studies have to be registered in the Personal Data Protection Act Register of the Personal Data Protection Commission. The influenza surveillance from which the clinical enterovirus D68 isolates were obtained is registered in this register and no further ethical approval was needed for this virologic study because only anonymized virus isolates were used.

Hemagglutination Assays. The hemagglutination assay was performed using standard methods. Briefly, twofold dilutions of the virus stocks were incubated with an erythrocyte suspension for 16 h at 4 °C.

ACKNOWLEDGMENTS. This work was supported by the Cancer Genomics Centre and EU FP7 Marie Curie Initial Training Network "EUVIRNA" Grant 264286 (to F.J.M.v.K.), the Netherlands Organization for Scientific Research Grants NWO-VICI-91812628 (to F.J.M.v.K.) and NWO-VIDI-91711316 (to T.R.B.), European Research Council ERC Starting Grant ERC-2012-StG 309634 (to T.R.B.), and National Institutes of Health Grant AI011219 (to M.G.R.).

1. Solomon T, et al. (2010) Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect Dis* 10(11):778–790.
2. Blomqvist S, Savolainen C, Råman L, Roivainen M, Hovi T (2002) Human rhinovirus 87 and enterovirus 68 represent a unique serotype with rhinovirus and enterovirus features. *J Clin Microbiol* 40(11):4218–4223.
3. Schieble JH, Fox VL, Lennette EH (1967) A probable new human picornavirus associated with respiratory diseases. *Am J Epidemiol* 85(2):297–310.
4. CDC (2011) Clusters of acute respiratory illness associated with human enterovirus 68-Asia, Europe, and United States, 2008-2010. *MMWR* 60(38):2008–2010.
5. Meijer A, et al. (2012) Emergence and epidemic occurrence of enterovirus 68 respiratory infections in The Netherlands in 2010. *Virology* 423(1):49–57.
6. Tokarz R, et al. (2012) Worldwide emergence of multiple clades of enterovirus 68. *J Gen Virol* 93(Pt 9):1952–1958.
7. Lauinger IL, et al. (2012) Lineages, sub-lineages and variants of enterovirus 68 in recent outbreaks. *PLoS One* 7(4):e36005.
8. Messacar K, et al. (2015) 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(9978):1662–1671.
9. Greninger AL, et al. (2015) A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012-14): A retrospective cohort study. *Lancet Infect Dis* 15(6):671–682.
10. Bergelson JM, Coyne CB (2013) Picornavirus entry. *Adv Exp Med Biol* 790:24–41.
11. Olson NH, et al. (1993) Structure of a human rhinovirus complexed with its receptor molecule. *Proc Natl Acad Sci USA* 90(2):507–511.
12. Liu Y, et al. (2015) Structure and inhibition of EV-D68, a virus that causes respiratory illness in children. *Science* 347(6217):71–74.
13. Uncapher CR, DeWitt CM, Colonno RJ (1991) The major and minor group receptor families contain all but one human rhinovirus serotype. *Virology* 180(2):814–817.
14. Imamura T, et al. (2014) Antigenic and receptor binding properties of enterovirus 68. *J Virol* 88(5):2374–2384.
15. Carette JE, et al. (2011) Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 477(7364):340–343.
16. Jae LT, et al. (2014) Virus entry. Lassa virus entry requires a trigger-induced receptor switch. *Science* 344(6191):1506–1510.
17. Carette JE, et al. (2009) Haploid genetic screens in human cells identify host factors used by pathogens. *Science* 326(5957):1231–1235.
18. Carette JE, et al. (2011) Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat Biotechnol* 29(6):542–546.
19. de Vries E, et al. (2012) Influenza A virus entry into cells lacking sialylated N-glycans. *Proc Natl Acad Sci USA* 109(19):7457–7462.
20. Stevenson RA, Huang J-A, Studdert MJ, Hartley CA (2004) Sialic acid acts as a receptor for equine rhinitis A virus binding and infection. *J Gen Virol* 85(Pt 9):2535–2543.
21. Shinya K, et al. (2006) Avian flu: Influenza virus receptors in the human airway. *Nature* 440(7083):435–436.
22. Nicholls JM, Bourne AJ, Chen H, Guan Y, Peiris JSM (2007) Sialic acid receptor detection in the human respiratory tract: Evidence for widespread distribution of potential binding sites for human and avian influenza viruses. *Respir Res* 8:73.
23. Wright PW, Strauss GH, Langford MP (1992) Acute hemorrhagic conjunctivitis. *Am Fam Physician* 45(1):173–178.
24. Kabue JP, et al. (2007) New enteroviruses, EV-93 and EV-94, associated with acute flaccid paralysis in the Democratic Republic of the Congo. *J Med Virol* 79(4):393–400.
25. Alexander DA, Dimock K (2002) Sialic acid functions in enterovirus 70 binding and infection. *J Virol* 76(22):11265–11272.
26. Liu Y, et al. (2015) Sialic acid-dependent cell entry of human enterovirus D68. *Nat Commun* 6:8865.
27. Powell RM, et al. (1998) Characterization of echoviruses that bind decay accelerating factor (CD55): Evidence that some haemagglutinating strains use more than one cellular receptor. *J Gen Virol* 79(Pt 7):1707–1713.
28. Hu L, et al. (2012) Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen. *Nature* 485(7397):256–259.
29. Malakhov MP, et al. (2006) Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. *Antimicrob Agents Chemother* 50(4):1470–1479.
30. Rhoden E, Zhang M, Nix WA, Oberste MS (2015) In vitro efficacy of antiviral compounds against enterovirus D68. *Antimicrob Agents Chemother* 59(12):7779–7781.
31. Jae LT, et al. (2013) Deciphering the glycosylome of dystroglycanopathies using haploid screens for lassa virus entry. *Science* 340(6131):479–483.
32. Dorobantu CM, et al. (2014) Recruitment of PI4KIIIβ to coxsackievirus B3 replication organelles is independent of ACBD3, GBF1, and Arf1. *J Virol* 88(5):2725–2736.
33. Belov GA, Fogg MH, Ehrenfeld E (2005) Poliovirus proteins induce membrane association of GTPase ADP-ribosylation factor. *J Virol* 79(11):7207–7216.
34. Lanke KHW, et al. (2009) GBF1, a guanine nucleotide exchange factor for Arf, is crucial for coxsackievirus B3 RNA replication. *J Virol* 83(22):11940–11949.