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1 **Early entry events in Echovirus 30 infection**

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38 **ABSTRACT**

39

40 Echovirus 30 (E30), a member of the enterovirus B species, is a major cause of viral meningitis,  
41 targeting children and adults alike. While it is a frequently isolated enterovirus and the cause of  
42 several outbreaks all over the world, suprisingly little is known regarding its entry and replication  
43 strategy within cells. In this study, we used E30 *Bastianni* (E30B) generated from an infectious cDNA  
44 clone in order to study early entry events during infection in human RD cells. E30B required the newly  
45 discovered Fc echovirus receptor (FcRn) for succesful infection, but not the Coxsackievirus and  
46 Adenovirus Receptor (CAR) or Decay-Accelerating Factor (DAF), although an interaction with DAF  
47 was observed. Double-stranded RNA replication intermediate was generated between 2 and 3 h post-  
48 infection (p.i.). and viral capsid production was initiated between 4 and 5 h p.i. The drugs affecting  
49 Rac1 (NSC 23766) and cholesterol (Filipin III) compromised infection, whereas bafilomycin A1, dyngo,  
50 U-73122, wortmannin and nocodazole did not, suggesting the virus follows an enterovirus-triggered  
51 macropinocytic pathway rather than the clathrin pathway. Colocalization with early endosomes and  
52 increased infection due to constitutively active Rab5 expression suggests some overlap and entry to  
53 classical early endosomes. Taken together, these results suggest that E30B induces an enterovirus  
54 entry pathway, leading to uncoating in early endosomes.

55 **IMPORTANCE**

56

57 Echovirus 30 (E30) is a prevalent enterovirus causing regular outbreaks in both children and adults in  
58 different parts of the world. It is therefore surprising that relatively little is known of its infectious entry  
59 pathway. We set out to generate a cDNA clone and gradient-purified the virus in order to study the  
60 early entry events in human cells. We have recently studied other enterovirus B group viruses, like  
61 echovirus 1 (EV1) and coxsackievirus A9 (CVA9), and found many similarities between those viruses,  
62 allowing us to define a so-called “enterovirus entry pathway”. Here, E30 is reminiscent of these  
63 viruses, e.g. by not relying on acidification for infectious entry. However, despite not using the clathrin  
64 entry pathway, E30 accumulates in classical early endosomes.

65 **KEYWORDS**  
66  
67 Enterovirus  
68 Echovirus 30  
69 Aseptic meningitis  
70 Early entry

## 71 INTRODUCTION

72

73 Meningeal inflammation lacking an identifiable bacterial origin is a common neurological syndrome  
74 known as aseptic meningitis. Its clinical course, however similar, is generally milder than that of its  
75 bacterial counterpart; nonetheless, viral meningitis occurs more frequently and leads to the  
76 hospitalisation of 26,000 to 42,000 people every year in the US alone, thus representing a significant  
77 economical and societal burden (1-3). Many different viruses can trigger the development of viral  
78 meningitis, such as herpesviruses, influenzaviruses, and arboviruses (4, 5). Since the introduction of  
79 the mumps, measles, and rubella (MMR) combination vaccine in 1988, however, non-polio  
80 enteroviruses have taken over as the leading cause of the disease, accounting for over 90 % of all  
81 cases in which the etiological agent has been identified (1, 4, 6-8). Among these, group B  
82 coxsackieviruses and echoviruses are the most commonly isolated types, in particular echovirus 30  
83 (E30) (8).

84 E30, a picornavirus belonging to the *Enterovirus B* genus, is a frequently isolated, positive-sense RNA  
85 virus of approximately 7,500 nucleotides enclosed by a non-enveloped protein capsid. Outbreaks of  
86 E30-related aseptic meningitis have been recorded every 3 – 5 years in many regions of the world,  
87 including Europe, Asia, and the United States (9-15). E30 is the enterovirus type that, over time, has  
88 been most frequently reported in humans with aseptic meningitis, and has been demonstrated to form  
89 a phylogenetic cluster with other notable echoviruses such as echovirus 21 (E21), echovirus 25 (E25),  
90 and echovirus 29 (E29) (2, 16). Moreover, the 5' noncoding region of E30 shows between 68 %  
91 (coxsackievirus A24, CVA24) and 93 % (coxsackievirus B3, CVB3) homology with other human  
92 enteroviruses, and appears to contain some coxsackie B-like genomic features (17). Despite often  
93 being the subject of medical and epidemiological reports, E30 has been grievously overlooked with  
94 regards to its life cycle and infection mechanisms. Using an infectious E30 *Bastianni* (E30B) cDNA  
95 clone, this project aimed to study early events in the echovirus life cycle, as well as to pinpoint key  
96 cellular components necessary for viral entry into the host cell. We show that E30B represents a  
97 typical enterovirus B group virus using an enterovirus-triggered macropinocytic entry pathway leading  
98 to rapid replication which does not require endosomal acidification to facilitate infection. However, it  
99 sets itself apart from its closest enterovirus relatives by showing accumulation in classical early  
100 endosomes. Being the first report detailing the mechanism of early entry and infection by E30B, this  
101 study may open the door to a deeper understanding of the life cycle and infection mechanisms of this  
102 pathogen.

103

## 104 RESULTS

105

106 **E30B displays efficient replication and infection kinetics in human RD cells.** E30B virus stocks  
107 were produced from a newly constructed cDNA clone, as described in the Materials and Methods.  
108 TEM visualisation of the negatively stained, gradient purified E30B revealed typical enteroviral  
109 particles, as E30B preparations consisted mainly of intact icosahedral viral particles with a low quantity  
110 of empty capsids (Fig. 1A,B). In order to visualise the entry and life cycle of E30B, we performed

111 immunolabeling of E30B infected RD and A549 cells during various time points post-infection (p.i.).  
112 Labeling of the replication intermediate using J2, an antibody specifically geared towards dsRNA,  
113 showed that the earliest signs of viral replication appeared between 2 and 3 h p.i. in both RD cells  
114 (Fig. 1C) and A549 cells (data not shown). Subsequent quantification of the dsRNA signal using a  
115 larger amount of cells from confocal images showed that dsRNA was already detectable at 2.5 h p.i.,  
116 and the intensity of the signal increased exponentially as the infection progressed (Fig. 1D).

117 Growth curve analysis through quantitative RT-PCR monitoring the E30B infection progression in RD  
118 cells also showed a low viral load before 3 h p.i., as evidenced by the high qRT-PCR cycle threshold  
119 (C<sub>t</sub>) value, followed by an increase in intracellular E30B RNA starting from 4 h p.i., confirming the  
120 dsRNA IF labeling (Fig. 1E). These results indicated that E30B adapted extremely well to RD cell  
121 culture and reached a high viral titre.

122 E30B infection was also followed using confocal microscopy by labeling the capsid with antibodies  
123 against VP1. E30B capsid protein could be visualised using the monoclonal rhesus monkey antiserum  
124 from ATCC originally prepared against human E30 virus (Fig. 2). The vesicular label was scattered  
125 and mostly peripheral for the first 4 h p.i., after which the cytoplasmic, more widespread signal  
126 increased. The labeling was also performed with the monoclonal mouse antiserum (Clone 5-D8/1,  
127 DAKO) reactive against several members of the enterovirus B group virus VP1 capsid proteins, which  
128 showed a similar distribution in infected cells (data not shown). Together, these data support the  
129 notion that E30B appears to exhibit similar efficient replication and infection kinetics as other members  
130 of the enterovirus B genus (18, 19).

131

132 **E30B uses DAF as its attachment receptor.** Members of the CVB cluster within the enterovirus B  
133 genus utilise the Coxsackievirus and Adenovirus receptor (CAR) to attach to and enter their respective  
134 host cells (20-22). While some enteroviruses also interact with the Decay-Accelerating Factor (DAF)  
135 receptor, this interaction in itself is often insufficient for virus entry into the cell (23-25). We performed  
136 a radioactive E30B receptor binding assay to assess the propensity for attachment of E30B to CAR  
137 and DAF. Based on this experiment, the virus does not bind to CAR but preferentially attaches to the  
138 DAF – receptor, a feature that is in line with previous findings (Fig. 3) (23, 26, 27). CHO-cells stably  
139 transfected with CAR (CHO-CAR) or DAF (CHO-DAF) were confirmed for strong DAF or CAR  
140 expression, respectively, using immunofluorescence and FACS (data not shown).

141 It has recently been shown that CD64, the Fc receptor (FcRn), acts as a pan-receptor for all  
142 echoviruses, including E30 (28, 29). We therefore performed a double labeling using antibodies  
143 against FcRn and capsid antibodies during E30B infection. Despite the presence of FcRn in A549  
144 cells, we found no difference in the distribution of the receptor in infected cells compared to non-  
145 infected cells. The receptor showed a small vesicular appearance in all studied timepoints. In addition,  
146 we found no apparent colocalization of E30B with FcRn at any infected timepoint (Fig. 4). This was  
147 confirmed by careful quantification of the colocalization using automatic thresholding for colocalization.  
148 The Manders' coefficient was kept at a maximum of 10 % in all studied timepoints, including in the  
149 non-infected control cells, suggesting that the colocalization was caused by the background noise.

150 As this was quite unexpected due to recent results on the importance of FcRn as an echoviral receptor  
151 also for E30B, we decided to perform siRNA knock-down of the receptors studied here: FcRn, DAF  
152 and CAR. Despite having no effect on the cellular distribution of FcRn during E30B infection, the  
153 siRNA treatment of FcRn completely abolished E30B infection in A549 cells (Fig. 5). In addition,  
154 siRNA knock-down of CAR or DAF, respectively, did not affect the infection of E30B. Furthermore, we  
155 used another enterovirus, namely CVB5, as a control, as it has been shown to use the CAR and DAF  
156 receptors, but not FcRn, during infection (21, 24, 29). Indeed, our results showed that, in contrast to  
157 E30B, CVB5 infection was not affected by FcRn knock-down, but instead, the infection was clearly  
158 decreased by CAR or DAF siRNA treatment (Fig. 5). Taken together, these results show that E30B  
159 requires FcRn for successful infection, although DAF, unlike CAR, may function as a co-receptor for  
160 attachment.

161

162 **Effects of pharmacological inhibitors on early E30B entry.** Other members of the enterovirus B  
163 subgroup have been previously shown to prefer the non-clathrin pathway to enter their respective host  
164 cells (22, 30-32). Through the use of chemical inhibitors known to affect the action of several key  
165 elements of cell entry, we attempted to assess which cellular components or pathways are  
166 indispensable for virus infection and replication to occur. Subconfluent monolayers of RD cells were  
167 treated with the different compounds for 30 min prior to virus addition, after which the infection was  
168 allowed to proceed for 6 h at 37 °C. After incubation, the cells were fixed and labeled with a pan-  
169 enteroviral VP1 capsid protein antibody. Immunofluorescent labeling of the viral capsid allowed visual  
170 distinction between infected and uninfected cells using confocal microscopy. The entry inhibitor drug  
171 NSC 23766 (inhibiting Rac1) drastically reduced the pathogen's capacity for infection, suggesting that  
172 this cellular components is essential for virus entry. In contrast, phosphoinositide 3-kinases (PI3K),  
173 endosomal acidification, phospholipase C activation, dynamin, and microtubule (de) polymerisation  
174 appeared not to influence E30B infection, as evidenced by the use of wortmannin, bafilomycin A1, U-  
175 73122, Dyngo 4a, and nocodazole, respectively (Fig. 6).

176 None of the compounds significantly affected cell survival in the used concentration compared to a  
177 control group, as evidenced by the evaluation of the cell toxicity assay (Fig. 7). This indicated the  
178 observed cell deaths were natural rather than a toxic chemical effect.

179 To confirm the confocal microscopy data, the capacity of these drugs to interfere with viral replication  
180 was further evaluated by quantification of the amount of intracellular viral RNA using qPCR (Fig. 8). As  
181 previously shown, NSC 23766 affected RNA replication, confirming our previous microscopy results.  
182 The treatment with NSC 23766 prevented RNA replication, resulting in an amount of viral RNA that  
183 was below the detectable level.

184 In addition, the effect of the cholesterol modifying drug Filipin III on E30B infection was studied using  
185 quantitative RT-PCR (Fig. 9A). The results showed that filipin treatment decreased replication, as the  
186  $C_t$  value increased from 20 for the control infection to 25 for the filipin treatment, corresponding to a  
187 30-fold decrease in viral RNA amount. The results also showed that the lowest effective concentration  
188 of filipin was not cytotoxic to RD cells (Fig. 9B).

189



190 **E30B colocalizes with early endosomes during early entry.** The early endosomes are a preferred  
191 sorting station for several incoming vesicles, regardless of the origin of the plasma membrane-derived  
192 vesicle. In our earlier studies with echovirus 1 (EV1) and coxsackievirus A9 (CVA9) we found  
193 negligible colocalization of the viruses with early endosomes (18, 33). Our results here with  
194 bafilomycin A1 and nocodazole showed that acidification of the endosomal structures and  
195 microtubule-dependent targeting of E30B to the perinuclear region was not necessary for infection.  
196 However, we were curious to find out if E30B would still enter the early sorting or early recycling  
197 endosomes; therefore, we infected RD cells with E30B and used confocal immunofluorescence  
198 microscopy to visualize possible colocalization of the virus with EEA1 for early sorting endosomes and  
199 the transferrin receptor for recycling early endosomes (Fig. 10). After an incubation period of 5 min  
200 colocalization of E30B with the endosomal markers was rather low; however, this colocalization  
201 increased dramatically after 30 min, suggesting that E30B does indeed invade the early endosomal  
202 compartments, but with delayed kinetics in comparison to cargo relying on clathrin-dependent entry.  
203 After 5 min, E30B colocalized to some extent both with internalized transferrin as well as with EEA1.  
204 Also, transferrin and EEA1 showed good colocalization of their signals, which is expected given that  
205 transferrin passes the sorting early endosomes on its way to recycling early endosomes. In contrast,  
206 as previously described, CVA9 did not colocalize with either of the endosomal markers, indicating that  
207 CVA9, unlike E30B, does not enter the early endosomal compartments at any time (18). Interestingly,  
208 after 30 min of E30B entry, there was much higher colocalization between transferrin and EEA1 and  
209 the volume of the colocalized structures had increased due to virus infection. This suggests that  
210 transferrin recycling and the overall dynamics of early endosomes were affected by the E30B  
211 infection.

212 Due to the involvement of early endosomes in E30B infection, we investigated the role of Rab5 in  
213 E30B infection by transfecting RD cells with different Rab5 constructs (Fig. 11). This small GTPase is  
214 important for the dynamics of early endosomes, particularly for their homotypic fusion (34). In addition,  
215 Rab5 and some of its effectors have been shown to regulate macropinosome dynamics (35, 36). Our  
216 experiment showed that E30B infection was approximately 40 % lower ( $p < 0.01$ ) in cells transfected  
217 with a dominant-negative Rab5 construct (pEGFP-Rab5-S34N) compared to the wild type Rab5  
218 control. In addition, overexpression of the constitutively active Rab5 gene markedly improved E30B  
219 infection as transfection with the constitutively active Rab5 (pEYFP-Rab5-Q79L) resulted in a circa 70  
220 % increase of infection compared to the wild type Rab5 ( $p < 0.05$ ). In contrast, the infection of CVA9  
221 was less affected by the dominant-negative Rab5 ( $p < 0.05$ ) and in comparison to E30B, constitutively  
222 active Rab5 decreased the infection of CVA9 which has also been previously shown (18). Altogether,  
223 these results further suggest that, in contrast to CVA9, E30B uses early endosomes as a route of  
224 entry.

225

## 226 DISCUSSION

227

228 Despite its role as a principal cause of viral meningitis, and the consequent extensive epidemiological  
229 and diagnostic attention it has received, the life cycle and replication mechanics of E30 have long

230 been overlooked. Here, we report the development of a novel infectious cDNA clone – the first of its  
231 kind, to the best of our knowledge – which actively replicates and infects in cell culture, allowing us to  
232 study and investigate early entry events in E30 infection using immunofluorescence and confocal  
233 microscopy.

234 Time-resolved analysis of the early infection progression suggested that E30B shows very similar  
235 infection and replication kinetics to other enterovirus B members, a notion that is supported by our  
236 findings regarding dsRNA production and initiation of viral replication (Fig. 1) (18, 19).

237 Members of the CVB cluster within the enterovirus B genus tend to favor the CAR – receptor to  
238 facilitate attachment and entry into their respective host cells (20-22). In some cases, the DAF –  
239 receptor may function as a coreceptor for attachment, but this interaction in itself is often insufficient to  
240 establish infection (23-25). We found that E30B does not bind to CAR and appears to attach to DAF  
241 on the plasma membrane, which is in accordance with previous findings (Fig. 3) (23, 26, 27). To our  
242 surprise, however, siRNA knock-down of DAF did not prevent the infection of E30B, indicating that  
243 although DAF promotes E30B binding to cells, it is not needed for infection. In contrast, the siRNA  
244 knock-down of FcRn prevented E30B infection despite the fact that the distribution of the receptor in  
245 the cytoplasm did not appear to be affected by E30B infection, nor did it colocalize with the virus after  
246 entry. Taken together, these results suggest that while DAF may facilitate the binding of E30B on cells  
247 and may function as a co-receptor, the FcRn receptor is absolutely required for successful infection,  
248 which is in accordance with previous studies (28, 29).

249 Early endosomes function as cellular sorting stations which accumulate various uptake vesicles from  
250 the plasma membrane. Delivery of these vesicles can occur via different routes, some of which can be  
251 hijacked by viruses to facilitate their entry into the host cell. The increased colocalization of E30B with  
252 both EEA1 and transferrin suggests that E30B does indeed accumulate in early endosomes. However,  
253 there were several lines of evidence to suggest that E30B does not use clathrin-dependent entry to  
254 early endosomes. First of all, inhibition of dynamin had no effect on infectivity. Second, the entry to the  
255 early endosomes took longer than the typical clathrin cargos, which accumulate in early endosomes  
256 within minutes. In addition, expression of the dominant-negative small GTPase Rab5 construct  
257 decreased E30B infection. Furthermore, transfection of a constitutively active Rab5 construct  
258 increased E30B infection, suggesting that an increased amount of homotypic fusion of early  
259 endosomes, and supposedly with other incoming vesicles, promoted E30B infection (Fig. 11). This led  
260 us to believe that E30B can make use of early endosomes, but does not rely on clathrin-dependent  
261 entry to facilitate its entry into these organelles.

262 In addition to entry, the results suggested that the progression of infection was not dependent on  
263 acidification, which is typical for the clathrin-dependent pathway. This was proven by the lack of an  
264 inhibitory effect of bafilomycin A1. Furthermore, the absence of an effect with nocodazole suggests  
265 there is no explicit need for endosomal acidification, microtubule transport to perinuclear regions and  
266 late endosomes, or recycling of early endosomes to establish infection. The results thus altogether  
267 indicate that entry into early endosomal structures occurs not via clathrin-coated pits, but rather  
268 through cholesterol-containing raft domains, following a longer route to reach its destination.

269 In conclusion, E30B proved to be a typical enterovirus by not relying on acidification to ensure  
270 infection. E30B showed a preference for DAF over CAR for cellular attachment, but demonstrated the  
271 Fc receptor to be an absolute requirement for infection. In contrast to EV1 and CVA9, E30B depends  
272 on sorting to early endosomes for efficient uncoating and infection. This study represents, to the best  
273 of our knowledge, the first in-depth examination of E30B early entry and virus-host cell interaction  
274 mechanics. The development of a viable, efficiently replicating E30B clone enables examination of the  
275 virus's life cycle and its behaviour *in vitro*, opening the door to the development of better treatment  
276 strategies and care.

277

## 278 MATERIALS & METHODS

279

280 **Cells and viruses.** Human RD and A549 cell lines, as well as CHO and GMK cells, were purchased  
281 from the American Tissue Culture Collection (ATCC). Additionally, two distinct lines of recombinant  
282 CHO cells (stably expressing human Coxsackie and Adenovirus receptor (CHO-CAR) and human  
283 Decay Accelerating Factor (CHO-DAF), respectively) were previously constructed by H. C. Selinka  
284 (37). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich)  
285 supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich), 1 % penicillin-streptomycin and 2  
286 mM L-glutamine (Sigma-Aldrich) at 37 °C, 5.0 % CO<sub>2</sub>.

287 An E30B infectious clone was designed using the prototype E30 *Bastianni* sequence (GenBank  
288 accession n° AF311938.1) and subsequently produced and cloned into a pUC57 cloning vector  
289 (GenScript). A previously described hammerhead ribozyme structure containing an inactivated AscI  
290 restriction enzyme site (G39C and C48G) was added at the 5' UTR, as well as a 28 A residue poly(A)  
291 tail at the 3' UTR (38). The plasmid was introduced into NovaBlue competent cells, which were  
292 incubated in LB-medium (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) and plated on LB/Amp (100  
293 µg/ml ampicillin) plates. Plasmid DNA was isolated and purified using the GeneJET Plasmid Miniprep  
294 Kit (Life Sciences).

295

296 **Transfection, virus production and purification.** RD cells were grown in a 6-well plate to 70 – 90 %  
297 confluence, transfected with E30B-pUC57 using Lipofectamine 2000 transfection reagent (InvitroGen),  
298 and incubated at 37 °C until complete cytopathic effect (CPE) was observed. Following transfection,  
299 cells were subjected to three rounds of freeze-thawing to ensure maximal virus yield, and generated  
300 viruses were further propagated through five serial passages to ensure adequate adaptation to the cell  
301 line. For each passage, 1.0 ml of lysate from either the transfection or the previous passage was  
302 added to subconfluent RD cells grown in a T25 flask, which was subsequently incubated for 1 h at  
303 room temperature. After incubation, the inoculum was removed, fresh cell medium was applied, and  
304 cells were further cultured at 37 °C until CPE was visible, or for a maximum of 5 days. To obtain  
305 purified virus, E30B was propagated in 5-layer flasks containing RD cells and subsequently purified  
306 using sucrose gradients as previously described (39). Cell culture medium for virus propagation and  
307 purification consisted of serum-free DMEM (Sigma-Aldrich) supplemented with 1 % penicillin-  
308 streptomycin and 2 mM L-glutamine (Sigma-Aldrich).

309

310 **Transmission electron microscopy.** Transmission electron microscopy (TEM) imaging was  
311 performed as previously described (40). Briefly, Butwar-coated copper grids were hydrophylised  
312 through glow discharging with an EMS/SC7620 Mini Sputter Coater (Quorum Technologies) as per the  
313 manufacturer's instructions before incubation with E30B for 15 s. Excess virus was removed, after  
314 which the remaining virions were negatively stained by incubating the grid with 1 % phosphotungstic  
315 acid in water (pH 7.4) for 1 min. After incubation, excess stain was removed and E30B (subjected to 5  
316 min of heat treatment at 50 °C prior to application) was added. Samples were dried overnight and  
317 subsequently evaluated using a JEM-1400 transmission electron microscope (JEOL).

318

319 **Confocal immunofluorescence imaging.** RD and A549 cells were cultured on coverslips to  
320 subconfluency, washed once with PBS, and subsequently infected with E30B. After incubation at 37  
321 °C, the cells were fixed at selected time points using 4 % paraformaldehyde (PFA) for 20 min,  
322 permeabilised using 0.2 % Triton X-100 for 5 min, and antibody labeled. The samples were mounted  
323 using Mowiol (Sigma-Aldrich) containing DABCO (1,4-diazabicyclo[2,2,2]octane, Sigma-Aldrich) and  
324 evaluated using an Olympus FluoView 1000 Laser Scanning Confocal Microscope or Leica SP8 with  
325 Leica's Lighting optimized settings using a voxel size of 35 nm in XY and 245 nm in Z.

326 The following antibodies were used: mouse monoclonal antisera against enterovirus VP1 capsid  
327 protein (cat. n° M7064, Dako), mouse monoclonal antisera against human EEA1 (cat. n° 610457, BD  
328 Biosciences), and mouse monoclonal antisera against dsRNA (J2, cat. n° 10010200, SCICONS);  
329 rabbit monoclonal antisera against Fc receptor (CD64, cat. n° ab193148, Abcam) and rabbit polyclonal  
330 antisera against EEA1 (cat. n° ab2900, Abcam); rhesus monkey monoclonal antiserum against human  
331 echovirus 30 (cat. n° VR-1072 AS/MK, ATCC); Alexa Fluor 488 goat polyclonal IgG against mouse  
332 (cat. n° A-11029, ThermoFisher Scientific); Alexa Fluor 555 goat polyclonal IgG against mouse (cat. n°  
333 A-21422, ThermoFisher Scientific); Alexa Fluor 488 goat polyclonal IgG against rabbit (cat. n° A-  
334 11008, ThermoFisher Scientific); Alexa Fluor 555 goat polyclonal IgG against rabbit (cat. n° A-21428,  
335 ThermoFisher Scientific); Alexa Fluor 647 goat polyclonal IgG against rhesus monkey (cat. n° 6200-31,  
336 SouthernBiotech). Antibody dilutions were prepared in 3 % bovine serum albumin (BSA) in PBS.

337

338 **Receptor binding assay.** Radioactive <sup>35</sup>S-labeled E30B was produced as previously described (40).  
339 Briefly, RD monolayers were grown to subconfluency, washed once with PBS, and infected for 3 h at  
340 37 °C with E30B diluted in low methionine/cysteine medium supplemented with 1 % FBS (Sigma-  
341 Aldrich). After incubation, the medium was replaced with low methionine/cysteine medium  
342 supplemented with 1 % FBS and 50 µCi/ml of [<sup>35</sup>S] methionine-cysteine (EasyTag EXPRESS <sup>35</sup>S  
343 Protein Labeling Mix [<sup>35</sup>S], PerkinElmer) and infection was allowed to continue for 9 h at 37 °C. Cell  
344 lysates were collected after repeated freeze-thaw cycles, after which cell debris was pelleted through  
345 centrifugation at 4 °C using an SL-16R rotor (2,500 × g for 10 min, ThermoFisher Scientific). The  
346 supernatant was incubated with 0.3 % (wt/vol) sodium deoxycholate (DOC) and 0.6 % (vol/vol)  
347 Nonidet P-40 (NP-40) substitute for 30 min on ice. Membrane structures were pelleted through  
348 centrifugation at 4 °C using an SL-16R rotor (4,000 × g for 10 min) and the supernatant was applied to

349 40 % sucrose cushions. Samples were ultracentrifuged at 4 °C using an SW-41 rotor (35,000 rpm for  
350 2.5 h, Beckman Coulter). The liquid above each cushion as well as one 500 µl fraction was discarded,  
351 while three subsequent 500 µl fractions were collected and applied to 5 – 20 % continuous sucrose  
352 gradients. Gradients were subjected to centrifugation at 4 °C using an SW-41 rotor (35,000 rpm for 2  
353 h) and fractioned into 500 µl aliquots starting from the top, which were consequently analyzed through  
354 addition of 4 ml of Ultima Gold MV scintillation cocktail (PerkinElmer) and application of the Liquid  
355 Scintillation Analyzer Tri-Carb 2910 TR scintillation counting method (PerkinElmer).

356 CHO cells stably transfected with CAR (CHO-CAR) or DAF (CHO-DAF) were tested for strong DAF or  
357 CAR expression by immunofluorescence and FACS. Each adherent cell culture was individually  
358 detached using trypsin (Sigma-Aldrich) before 150,000 cells per replicate were washed, resuspended  
359 in 2 mM MgCl<sub>2</sub>-PBS, and subsequently incubated at 4 °C with 50,000 CPM of <sup>35</sup>S-labeled E30B  
360 (corresponding to MOI 850). After 1 hour, cells were washed to remove unbound virions, resuspended  
361 in 4 ml of Ultima Gold MV scintillation cocktail (PerkinElmer), and analyzed using the Liquid  
362 Scintillation Analyzer Tri-Carb 2910 TR scintillation counting method (PerkinElmer).

363

364 **siRNA transfections.** A549 cells were reverse transfected using DharmaFECT transfection reagent  
365 (Horizon Discovery) according to the manufacturer's instructions. The pool of three target-specific  
366 siRNAs against CAR, DAF or FcRn (Santa Cruz) or AllStars negative control siRNA (kindly gifted by  
367 the Johanna Ivaska laboratory, University of Turku, Turku, Finland) were added in a final  
368 concentration of 11.4 nM and the transfection was allowed to proceed for 48 h at 37 °C in DMEM  
369 supplemented with 10 % FBS and 1 % GlutaMax. Next, 200 PFU/cell of E30B or coxsackievirus B5  
370 (CVB5) were added in DMEM supplemented with 1 % FBS and 1 % GlutaMax and bound on ice for 1  
371 h after which the excess virus was washed away (41). The infection was then allowed to proceed at 37  
372 °C in DMEM supplemented with 10 % FBS, 1 % GlutaMax for 6 h after which the cells were collected  
373 into 2 X Laemmli buffer containing β-mercaptoethanol.

374

375 **SDS-PAGE and Western blot.** The samples were boiled and separated in a 4-20% Mini-PROTEAN  
376 TGX Stain-Free gel (BioRad). Next, the proteins were transferred to PVDF membranes (Millipore) and  
377 blocked overnight with 5 % BSA and 0.05 % Tween in TBS. Blots were immunolabeled with mouse  
378 monoclonal antisera against enterovirus VP1 capsid protein (cat. n° M7064, Dako), and mouse  
379 monoclonal antisera against γ-tubulin (Abcam) was used as a loading control. The primary antibodies  
380 were detected using corresponding horseradish peroxidase-conjugated secondary antibodies (Cell  
381 Signaling). Finally, the chemiluminescent substrate SuperSignal West Pico PLUS (ThermoFisher  
382 Scientific) was incubated for 5 min and chemiluminescence was detected using the ChemiDoc MP  
383 (BioRad).

384

385 **Pharmacological inhibitor assay.** RD cells were cultured on coverslips to subconfluency. The cells  
386 were washed once with PBS and subsequently incubated at 37.0 °C in DMEM supplemented with  
387 50.0 nM Bafilomycin A1 (targeting vacuolar type H<sup>+</sup> – ATPase, cat. n° 196000, Calbiochem), 33.0 µM  
388 Nocodazole (affecting microtubule assembly/disassembly, cat. n° 487928, Calbiochem), 100.0 µM

389 Wortmannin (inhibiting phosphoinositide 3-kinase, cat. n° 681675, Calbiochem), 1.0 mM NSC 23766  
390 (targeting Rac1, cat. n° 2161, Tocris BioScience), 10.0 µM U-73122 (affecting phospholipase C, cat.  
391 n° 662035, Calbiochem), or 12.5 µM Dyngo 4a (inhibiting dynamin, cat. n° 120689, Abcam) for 30 min  
392 prior to E30B addition (18). The infection assay was carried out for 6 h at 37 °C, after which the cells  
393 were fixed using 4 % PFA for 20 min, antibody labeled, and the presence of virus capsid protein was  
394 determined.

395 The cellular toxicity of the pharmacological inhibitors was evaluated using the CellTiter-Glo  
396 Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol.

397

398 **Filipin assay.** RD cells were cultured until subconfluency and incubated for 30 min with a 1, 2 or 3  
399 µg/ml concentration of Filipin III (cat n° F4767, Sigma-Aldrich). Next, E30B was added onto the cells  
400 (200 PFU/cell) and bound on ice for 1 h after which excess virus was washed away. The infection was  
401 allowed to proceed for 6 h in DMEM supplemented with 10 % FBS, 1 % penicillin-streptomycin and 1  
402 % GlutaMax, also including 1, 2 or 3 µg/ml of filipin. Finally, the medium was removed and viral RNA  
403 was isolated from lysed cells using the QIAamp Viral RNA extraction kit (Qiagen) according to the  
404 manufacturer's instructions. Reverse transcription was carried out for positive-sense RNA using 1.2  
405 µM antisense primer (5'-GAAACACGGACACCCAAAGTA-3'), 20 U M-MLV Reverse Transcriptase  
406 (Promega), dNTPs (Promega) and 4 U RNasin Ribonuclease Inhibitor (Promega). 5 µl from the 40 µl  
407 reaction mixture was subsequently used in a PCR reaction which also included SYBR Green  
408 Supermix (BioRad) and 600 nM of both antisense primer (5'-GAAACACGGACACCCAAAGTA-3') and  
409 sense primer (5'-CGGCCCTGAATGCGGCTAA-3'). The amplification was carried out on the C1000  
410 Touch Thermal Cycler with CFX96 Touch Real-Time PCR Detection System (BioRad) using the  
411 following protocol: 95 °C for 10 min; 40 cycles of 95 °C for 15 s to 60 °C for 1 min; and final melting  
412 step at 72 to 95 °C, 1 °C / 5 s. The assay also contained negative controls to confirm the specificity of  
413 the products.

414 The cytotoxicity of filipin was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay  
415 (Promega) according to the manufacturer's protocol.

416

417 **Quantification of viral infection.** Viral RNA was extracted from infected RD cell cultures using the  
418 QIAamp Viral RNA extraction kit (Qiagen) according to the manufacturer's protocol and subsequently  
419 copied to cDNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems). Two-  
420 step qRT-PCR reactions were carried out on the 7500 Real-Time PCR System (Applied Biosystems)  
421 with 7500 SDS analysis software. Each reaction was prepared using 1 µl cDNA, 1 X Power SYBR  
422 Green Master Mix (Applied Biosystems), and 200 nM each of primers 5UTR-F (5'-  
423 CGTTGCGGAGTGTTTCGTTTC-3') and 5UTR-R (5'-TCCGCAGTTAGGATTAGCCG-3') directed  
424 against the 5' UTR of the genome in a final reaction volume of 20 µl. The following thermocycling  
425 program was applied: reverse transcription at 50 °C for 2 min, Taqman<sup>®</sup> DNA polymerase activation  
426 and simultaneous reverse transcriptase inactivation at 95 °C for 10 min, followed by 40 cycles of 15 s.  
427 at 95 °C and 60 s. at 60 °C. Each reaction was run in triplicate. Standard curves were generated by  
428 running the aforementioned protocol using the E30B cDNA template in triplicate.

429

430 **Transferrin – EEA1 assay.** RD cells were cultured on coverslips to subconfluency, washed once with  
431 PBS, and incubated with purified E30B or CVA9 (Griggs strain) for 1 h on ice. After virus binding, the  
432 medium was removed, cells were gently washed with 0.5 % BSA – PBS, and 50 µg/ml of transferrin –  
433 Alexa Fluor 488 conjugate (transferrin from human serum, Alexa Fluor 488 conjugate, InvitroGen) in  
434 DMEM supplemented with 0.2 % BSA was added. Infection was carried out at 37 °C and terminated at  
435 5 min and 30 min timepoints by fixing the cells with 4 % PFA for 20 min, after which the cells were  
436 antibody labeled and imaged.

437

438 **Plasmid transfections.** RD cells were grown on coverslips to subconfluency. Plasmid transfections  
439 were carried out for 48 h at 37 °C using Lipofectamine 3000 transfection reagent (InvitroGen)  
440 according to the manufacturer's instructions. The cells were infected with 200 PFU/cell of E30B or  
441 CVA9 (Griggs strain) by binding the virus on ice for 1 h in DMEM supplemented with 1 % FBS and 1  
442 % GlutaMax. After excess virus was washed away, the infection was allowed to proceed for 6 h at 37  
443 °C in DMEM supplemented with 10 % FBS, 1 % GlutaMax, and 1 % penicillin-streptomycin. Finally,  
444 the cells were fixed using 4 % PFA for 20 min and labeled with mouse monoclonal antisera against  
445 enterovirus VP1 capsid protein (cat. n° M7064, Dako). The infection percentage of transfected cells  
446 was quantified by evaluating the presence of viral capsid protein.

447 Plasmid constructs were obtained from the following sources: the dominant-negative (pEGFP-Rab5-  
448 S34N) and dominant-positive (pEYFP-Rab5-Q79L) Rab5 constructs were procured from Lucas  
449 Pelkmans (Department of Molecular Life Sciences, University of Zurich, Zurich, Switzerland), and the  
450 wild type Rab5 (pEGFP-Rab5) was acquired from Miguel Seabra (Faculty of Medicine, National Heart  
451 and Lung Institute, Imperial College, London, United Kingdom).

452

453 **Microscopy data analysis.** Microscope settings were optimised for each channel prior to imaging.  
454 Confocal immunofluorescence image analysis was executed using the Fiji free open source software  
455 package (42). For the colocalization analysis, the coloc2 plugin was used to measure the Manders'  
456 correlation and Costes' significance with a PSF estimation of 8 pixels and 20 iterations (43, 44). Image  
457 analysis was executed using the Fiji free open source software package (42). To visualize colocalizing  
458 pixels between transferrin, EEA1 and E30B or CVA9 (Griggs strain) the open source software  
459 BioImageXD ([www.bioimagexd.net](http://www.bioimagexd.net)) was used. Thresholding for E30B was set with the help of  
460 uninfected controls, and for transferrin and EEA1 they were set manually, to not contain background  
461 signal.

462

463 **Statistical analysis.** Statistical sample comparison of proportions and ratios was performed using an  
464 arcsine square root transformation to convert the data to be more normally distributed, followed by a  
465 paired or unpaired t-test. A p-value of < 0.05 was considered statistically significant.

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467

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471

472 **CONFLICTS OF INTEREST**

473

474 None to declare.



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- 586

587 **FIGURE LEGENDS**

588

589 FIG. 1. E30B displays efficient replication and infection kinetics. (A, B) Transmission electron  
590 microscope imaging of purified virus particles. Scale bars are 500 nm (A) and 100 nm (B). (C)  
591 Immunofluorescence staining of RD cells, 2 h, 3 h and 7 h post-infection (p.i.) with E30B. The  
592 presence of double-stranded RNA (dsRNA) indicates viral replication. Red, dsRNA (J2); blue, nuclei  
593 (DAPI). Scale bar is 15  $\mu$ m. (D) Time-resolved quantification of intracellular dsRNA accumulation in  
594 RD cells after E30B infection through measurement of the anti-dsRNA (J2) signal intensity. Results  
595 are presented as mean values of 12 areas containing 5 – 6 cells each ( $\pm$  standard error of the mean  
596 [SEM]) (E) Time-resolved quantitative RT-PCR following the intracellular accumulation of E30B RNA  
597 in RD cells. A high cycle threshold ( $C_t$ ) value corresponds to a low amount of intracellular viral RNA.  
598 Results are presented as mean values of 3 replicates ( $\pm$  standard deviation [SD]).

599

600 FIG. 2. Immunofluorescence staining of the E30B capsid in infected RD cells. Infected cells are  
601 marked with an asterisk (\*). Purple, VP1 capsid protein (antibody made in rhesus monkeys); blue,  
602 nuclei (DAPI). Scale bar is 10  $\mu$ m.

603

604 FIG. 3. E30B binding assay. 50,000 counts per minute (CPM) of metabolically labeled E30B was  
605 bound on ice to 150,000 CHO cells for 1 hour and washed. Results are presented as mean values of 3  
606 replicates ( $\pm$  standard error of the mean [SEM]). CHO-cells stably transfected with CAR (CHO-CAR) or  
607 DAF (CHO-DAF) were tested for strong DAF or CAR expression by immunofluorescence and FACS  
608 (data not shown).

609

610 FIG. 4. Colocalization analysis of E30B and FcRn in A549 cells. Example of the localisation of E30B  
611 and FcRn signals from representative cells for each time point shown as a maximum intensity  
612 projection. The Manders' coefficient represents the percentage of E30B voxels colocalizing with the  
613 FcRn voxels.

614

615 FIG. 5. The effect of CAR, DAF and FcRn siRNA knock-down on E30B infection. A549 cells were  
616 transfected for 48 h with pooled siRNAs against CAR, DAF or FcRn or with negative control siRNA  
617 (CTRLsi) and infected with E30B or CVB5 for 6 h. Top, representative image of Western blot where  
618 the infection was detected by immunolabeling of VP1 and  $\gamma$ -tubulin as a loading control. Bottom,  
619 quantification of the infection from Western blots by normalizing the VP1 signal to  $\gamma$ -tubulin. Results  
620 are presented as mean values of 3 replicates ( $\pm$  standard error of the mean [SEM]). Statistical  
621 significance was determined using an unpaired t-test. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

622

623 FIG. 6. Pharmacological inhibition of early E30B entry. Immunofluorescence staining of RD cells, 6 h  
624 post-infection (p.i.) with E30B. Cells were pretreated with inhibitory chemicals 30 min before addition

625 of the virus. The presence of capsid indicates viral replication. Red, viral capsid (DAKO); blue, nuclei  
626 (DAPI). Scale bar is 20  $\mu$ m.

627

628 FIG. 7. Cell viability assay of RD cells treated with pharmacological compounds. Cell viability  
629 measurement of RD cells, 6.5 h after treatment with indicated inhibitory chemicals. Results are  
630 presented as mean values of 6 replicates ( $\pm$  standard deviation [SD]). CTRL, untreated control cells.

631

632 FIG. 8. Pharmacological inhibition of early E30B entry. Quantitative RT-PCR measuring the  
633 intracellular accumulation of E30B RNA in RD cells treated with pharmacological inhibitors, 6 h post-  
634 infection (p.i.). Cells were pretreated with inhibitory chemicals 30 min before addition of the virus. A  
635 high cycle threshold ( $C_t$ ) value corresponds to a low amount of intracellular viral RNA. Results are  
636 presented as mean values of 3 replicates ( $\pm$  standard deviation [SD]). POS, positive control for  
637 infection without the presence of the vehicle; NEG, negative control for infection; DMSO: positive  
638 control for infection in the presence of the vehicle.

639

640 FIG. 9. The effect of the cholesterol modifying drug filipin on E30B infection. (A) Quantitative RT-PCR  
641 measuring the intracellular accumulation of E30B RNA in RD cells treated with different concentrations  
642 of the caveolae pathway inhibitor filipin, 6 h post-infection (p.i.). Results are presented as mean values  
643 of 3 replicates ( $\pm$  standard deviation [SD]). CTRL E30B, control infection without filipin. Statistical  
644 significance was determined using an unpaired t-test. \*\*\*\*,  $p < 0.0001$ . (B) Cell viability assay of RD  
645 cells after treatment with different filipin concentrations for 6 h. Results are presented as mean values  
646 of 3 replicates ( $\pm$  standard deviation [SD]). CTRL, untreated control cells.

647

648 FIG. 10. E30B colocalizes with early endosomes during early entry. E30, or CVA9 as a control, was  
649 bound to RD cells on ice, washed, and incubation was continued at 37  $^{\circ}$ C with transferrin – Alexa  
650 Fluor 488 for 5 or 30 min, after which cells were fixed and labeled also for EEA1. Green, transferrin  
651 receptor (transferrin – Alexa Fluor 488); red, early endosomal antigen (EEA1); purple, VP1 capsid  
652 protein (antibody made in rhesus monkeys). Scale bar is 10  $\mu$ m.

653

654 FIG. 11. The effect of Rab5 on E30B infection. RD cells were transfected with a wild type (pEGFP-  
655 Rab5-WT), a dominant-negative (pEGFP-Rab5-S34N) or a constitutively active (pEGFP-Rab5-Q79L)  
656 Rab5 construct. Cells and plasmids were incubated for 48 h to allow for stable expression, followed by  
657 infection for 6 h with E30B or CVA9, after which the infection was detected using immunofluorescence  
658 microscopy. The infection percentage of transfected cells was quantified based on VP1 signal, and  
659 350-550 transfected cells were calculated per sample in total. Results are normalized to wild type  
660 control and presented as mean values of 3 replicates ( $\pm$  standard error of the mean [SEM]). Statistical  
661 significance was determined using an arcsine square root transformation followed by an unpaired t-  
662 test. \*,  $p < 0.05$ ; \*\*  $p < 0.01$ .





























