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Early entry events in Echovirus 30 infection

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38 ABSTRACT

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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 Rac1 (NSC 23766) and cholesterol (Filipin III) compromised infection, whereas bafilomycin A1, dyngo, 49 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 classical early endosomes. Taken together, these results suggest that E30B induces an enterovirus 53 54 entry pathway, leading to uncoating in early endosomes.

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55 **IMPORTANCE**

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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.

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65 **KEYWORDS** 66

- 67 Enterovirus
- 68 Echovirus 30
- 69 Aseptic meningitis
- 70 Early entry

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71 INTRODUCTION

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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 cases in which the etiological agent has been identified (1, 4, 6-8). Among these, group B 81 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.

104 **RESULTS**

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

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

Growth curve analysis through quantitative RT-PCR monitoring the E30B infection progression in RD cells also showed a low viral load before 3 h p.i., as evidenced by the high qRT-PCR cycle threshold (C₁) value, followed by an increase in intracellular E30B RNA starting from 4 h p.i., confirming the 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).

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).

It has recently been shown that CD64, the Fc receptor (FcRn), acts as a pan-receptor for all 141 echoviruses, including E30 (28, 29). We therefore performed a double labeling using antibodies 142 143 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, we found no apparent colocalization of E30B with FcRn at any infected timepoint (Fig. 4). This was 146 147 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.

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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 siRNA treatment of FcRn completely abolished E30B infection in A549 cells (Fig. 5). In addition, 153 154 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 E30B, CVB5 infection was not affected by FcRn knock-down, but instead, the infection was clearly 157 decreased by CAR or DAF siRNA treatment (Fig. 5). Taken together, these results show that E30B 158 159 requires FcRn for successful infection, although DAF, unlike CAR, may function as a co-receptor for 160 attachment.

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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 cells (22, 30-32). Through the use of chemical inhibitors known to affect the action of several key 164 165 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.

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

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

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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 endosomes; therefore, we infected RD cells with E30B and used confocal immunofluorescence 197 198 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.

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226 DISCUSSION

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

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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.

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278 MATERIALS & METHODS

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Cells and viruses. Human RD and A549 cell lines, as well as CHO and GMK cells, were purchased from the American Tissue Culture Collection (ATCC). Additionally, two distinct lines of recombinant CHO cells (stably expressing human Coxsackie and Adenovirus receptor (CHO-CAR) and human Decay Accelerating Factor (CHO-DAF), respectively) were previously constructed by H. C. Selinka (37). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich), 1 % penicillin-streptomycin and 2 mM L-glutamine (Sigma-Aldrich) at 37 °C, 5.0 % CO₂.

287 An E30B infectious clone was designed using the prototype E30 Bastianni sequence (GenBank 288 accession nº AF311938.1) and subsequently produced and cloned into a pUC57 cloning vector 289 (GenScript). A previously described hammerhead ribozyme structure containing an inactivated Ascl 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).

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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).

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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 subsequently evaluated using a JEM-1400 transmission electron microscope (JEOL). 317

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Confocal immunofluorescence imaging. RD and A549 cells were cultured on coverslips to subconfluency, washed once with PBS, and subsequently infected with E30B. After incubation at 37 °C, the cells were fixed at selected time points using 4 % paraformaldehyde (PFA) for 20 min, permeabilised using 0.2 % Triton X-100 for 5 min, and antibody labeled. The samples were mounted using Mowiol (Sigma-Aldrich) containing DABCO (1,4-diazabicyclo[2,2,2]octane, Sigma-Aldrich) and evaluated using an Olympus FluoView 1000 Laser Scanning Confocal Microscope or Leica SP8 with 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. nº M7064, Dako), mouse monoclonal antisera against human EEA1 (cat. nº 610457, BD 328 Biosciences), and mouse monoclonal antisera against dsRNA (J2, cat. n° 10010200, SCICONS); rabbit monoclonal antisera against Fc receptor (CD64, cat. nº ab193148, Abcam) and rabbit polyclonal 329 330 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° A-21422, ThermoFisher Scientific); Alexa Fluor 488 goat polyclonal IgG against rabbit (cat. n° A-333 334 11008, ThermoFisher Scientific); Alexa Fluor 555 goat polyclonal IgG against rabbit (cat. n° A-21428, 335 ThermoFisher Scientific); Alexa Fluor 647 goat polyclonal IgG agains rhesus monkey (cat. n° 6200-31, SouthernBiotech). Antibody dilutions were prepared in 3 % bovine serum albumin (BSA) in PBS. 336

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

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

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

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 supplemented with 10 % FBS and 1 % GlutaMax. Next, 200 PFU/cell of E30B or coxsackievirus B5 369 (CVB5) were added in DMEM supplemented with 1 % FBS and 1 % GlutaMax and bound on ice for 1 370 371 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.

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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).

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

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

The cellular toxicity of the pharmacological inhibitors was evaluated using the CellTiter-Glo 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 sense primer (5'-CGGCCCCTGAATGCGGCTAA-3'). The amplification was carried out on the C1000 409 410 Touch Thermal Cycler with CFX96 Touch Real-Time PCR Detection System (BioRad) using the following protocol: 95 °C for 10 min; 40 cycles of 95 °C for 15 s to 60 °C for 1 min; and final melting 411 412 step at 72 to 95 °C, 1 °C / 5 s. The assay also contained negative controls to confirm the specificity of 413 the products.

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

416

Quantification of viral infection. Viral RNA was extracted from infected RD cell cultures using the 417 418 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 gRT-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 CGTTGCGGAGTGTTTCGTTC-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 program was applied: reverse transcription at 50 °C for 2 min, Tagman[®] DNA polymerase activation 425 and simultaneous reverse transcriptase inactivation at 95 °C for 10 min, followed by 40 cycles of 15 s. 426 427 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.

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429

Transferrin – EEA1 assay. RD cells were cultured on coverslips to subconfluency, washed once with PBS, and incubated with purified E30B or CVA9 (Griggs strain) for 1 h on ice. After virus binding, the medium was removed, cells were gently washed with 0.5 % BSA – PBS, and 50 µg/ml of transferrin – Alexa Fluor 488 conjugate (transferrin from human serum, Alexa Fluor 488 conjugate, InvitroGen) in DMEM supplemented with 0.2 % BSA was added. Infection was carried out at 37 °C and terminated at 5 min and 30 min timepoints by fixing the cells with 4 % PFA for 20 min, after which the cells were 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. nº M7064, Dako). The infection percentage of transfected cells 446 was quantified by evaluating the presence of viral capsid protein.

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

452

Microscopy data analysis. Microscope settings were optimised for each channel prior to imaging. 453 454 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' correlation and Costes' significance with a PSF estimation of 8 pixels and 20 iterations (43, 44). Image 456 457 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 BioImageXD (www.bioimagexd.net) was used. Thresholding for E30B was set with the help of 459 460 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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472 CONFLICTS OF INTEREST

- 473
- 474 None to declare.

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475 **REFERENCES**

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- 1. Irani DN. 2008. Aseptic meningitis and viral myelitis. Neurol Clin 26:635-655.
- 478 2. Khetsuriani N, Quiroz ES, Holman RC, Anderson LJ. 2003. Viral meningitis-associated
 479 hospitalizations in the United States, 1988-1999. Neuroepidemiology 22:345-352.
- Pallansch MA, Roos RP. 2007. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and
 newer enteroviruses, p 839-893. *In* Knipe DM, Howley PM (ed), Fields Virology, 5th ed, vol 1.
 Lippincott Williams & Wilkins, a Wolters Kluwer business, Philadelphia, USA.
- 483 4. Connolly KJ, Hammer SM. 1990. The acute aseptic meningitis syndrome. Infect Dis Clin North
 484 Am 4:599-622.
- 485 5. Centers for Disease Control and Prevention (CDC). 2016. Viral meningitis.
 486 <u>https://www.cdc.gov/meningitis/viral.html</u>. Accessed 03 Dec 2018.
- 487 6. Rotbart HA. 2000. Viral meningitis. Semin Neurol 20:277-292.
- 488 7. Davidson KL, Ramsay ME. 2003. The epidemiology of acute meningitis in children in England
 489 and Wales. Arch Dis Child 88:662-664.
- 490 8. Logan SA, MacMahon E. 2008. Viral meningitis. BMJ 336:36-40.
- Yamashita K, Miyamura K, Yamadera S, Kato N, Akatsuka M, Hashido M, Inouye S, Yamazaki S.
 1994. Epidemics of aseptic meningitis due to echovirus 30 in Japan. A report of the National
 Epidemiological Surveillance of Infectious Agents in Japan. Jpn J Med Sci Biol 47:221-239.
- 494 10. Oberste MS, Maher K, Kennett ML, Campbell JJ, Carpenter MS, Schnurr D, Pallansch MA. 1999.
 495 Molecular epidemiology and genetic diversity of echovirus type 30 (E30): genotypes correlate
 496 with temporal dynamics of E30 isolation. J Clin Microbiol 37:3928-3933.
- 497 11. Thoelen I, Lemey P, Van Der Donck I, Beuselinck K, Lindberg AM, Van Ranst M. 2003. Molecular
 498 typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in
 499 Belgium during the summer of 2000. J Med Virol 70:420-429.
- 500 12. Centers for Disease Control and Prevention (CDC). 2003. Outbreaks of aseptic meningitis
 501 associated with echoviruses 9 and 30 and preliminary surveillance reports on enterovirus
 502 activity—United States, 2003. MMWR Morb Mortal Wkly Rep 52:761-764.
- 503 13. Centers for Disease Control and Prevention (CDC). 2006. Enterovirus surveillance—United
 504 States, 2002-2004. MMWR Morb Mortal Wkly Rep 55:153-156.
- McWilliam Leitch EC, Bendig J, Cabrerizo M, Cardosa J, Hyypiä T, Ivanova OE, Kelly A, Kroes
 AC, Lukashev A, MacAdam A, McMinn P, Roivainen M, Trallero G, Evans DJ, Simmonds P.
 2009. Transmission networks and population turnover of echovirus 30. J Virol 83:2109-2118.
- 508 15. Broberg EK, Simone B, Jansa J, The EU/EEA Member State Contributors. 2018. Upsurge in
 509 echovirus 30 detections in five EU/EEA countries, April to September, 2018. Euro Surveill 23.
- 510 16. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA. 1999. Molecular evolution of the human
 511 enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus
 512 classification. J Virol 73:1941-1948.
- 513 17. Diedrich S, Driesel G, Schreier E. 1995. Sequence comparison of echovirus type 30 isolates to
 514 other enteroviruses in the 5' noncoding region. J Med Virol 46:148-152.

- 517 19. Pietiäinen V, Marjomäki V, Upla P, Pelkmans L, Helenius A, Hyypiä T. 2004. Echovirus 1
 518 endocytosis into caveosomes requires lipid rafts, dynamin II, and signaling events. Mol Biol Cell
 519 15:4911-4925.
- 520 20. Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS,
 521 Crowell RL, Finberg RW. 1997. Isolation of a common receptor for coxsackie B viruses and
 522 addenoviruses 2 and 5. Science 275:1320-1323.
- 523 21. Martino TA, Petric M, Weingartl H, Bergelson JM, Opavsky MA, Richardson CD, Modlin JF,
 524 Finberg RW, Kain KC, Willis N, Gauntt CJ, Liu PP. 2000. The coxsackie-adenovirus receptor
 525 (CAR) is used by reference strains and clinical isolates representing all six serotypes of
 526 coxsackievirus group B and by swine vesicular disease virus. Virology 271:99-108.
- 527 22. Marjomäki V, Turkki P, Huttunen M. 2015. Infectious entry pathway of enterovirus B species.
 528 Viruses 7:6387-6399.
- 529 23. Bergelson JM, Chan M, Solomon KR, St John NF, Lin H, Finberg RW. 1994. Decay-accelerating
 530 factor (CD55), a glycosylphosphatidylinositol-anchored complement regulatory protein, is a
 531 receptor for several echoviruses. Proc Natl Acad Sci U S A 91:6245-6248.
- 532 24. Shafren DR, Bates RC, Agrez MV, Herd RL, Burns GF, Barry RD. 1995. Coxsackieviruses B1,
 533 B3, and B5 use decay accelerating factor as a receptor for cell attachment. J Virol 69:3873-3877.
- 534 25. Milstone AM, Petrella J, Sanchez MD, Mahmud M, Whitbeck JC, Bergelson JM. 2005. Interaction
 535 with coxsackievirus and adenovirus receptor, but not with decay-accelerating factor (DAF),
 536 induces A-particle formation in a DAF-binding coxsackievirus B3 isolate. J Virol 79:655-660.
- 537 26. Powell RM, Schmitt V, Ward T, Goodfellow I, Evans DJ, Almond JW. 1998. Characterization of
 538 echoviruses that bind decay accelerating factor (CD55): evidence that some haemagglutinating
 539 strains use more than one cellular receptor. J Gen Virol 79:1707-1713.
- Rothe D, Werk D, Niedrig S, Horbelt D, Grunert HP, Zeichhardt H, Erdmann VA, Kurreck J. 2009.
 Antiviral activity of highly potent siRNAs against echovirus 30 and its receptor. J Virol Methods
 157:211-218.
- 543 28. Morosky S, Wells AI, Lemon K, Evans AS, Schamus S, Bakkenist CJ, Coyne CB. 2019. The
 544 neonatal Fc receptor is a pan-echovirus receptor. Proc Natl Acad Sci U S A 116:3758-3763.
- Zhao X, Zhang G, Liu S, Chen X, Peng R, Dai L, Qu X, Li S, Song H, Gao Z, Yuan P, Liu Z, Li C,
 Shang Z, Li Y, Zhang M, Qi J, Wang H, Du N, Wu Y, Bi Y, Gao S, Shi Y, Yan J, Zhang Y, Xie Z,
 Wei W, Gao GF. 2019. Human neonatal Fc receptor is the cellular uncoating receptor for
 enterovirus B. Cell 177:1553-1565.
- Triantafilou K, Triantafilou M. 2004. Lipid-raft-dependent coxsackievirus B4 internalization and
 rapid targeting to the Golgi. Virology 326:6-19.
- 31. Patel KP, Coyne CB, Bergelson JM. 2009. Dynamin- and lipid raft-dependent entry of decayaccelerating factor (DAF)-binding and non-DAF-binding coxsackieviruses into nonpolarized cells.
 J Virol 83:11064-11077.

554 32. Krieger SE, Kim C, Zhang L, Marjomaki V, Bergelson JM. 2013. Echovirus 1 entry into polarized
555 Caco-2 cells depends on dynamin, cholesterol, and cellular factors associated with
556 macropinocytosis. J Virol 87:8884-8895.

- 33. Marjomäki V, Pietiäinen V, Matilainen H, Upla P, Ivaska J, Nissinen L, Reunanen H, Huttunen P,
 Hyypiä T, Heino J. 2002. Internalization of echovirus 1 in caveolae. J Virol 76:1856-1865.
- 34. Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M. 1992. The small
 GTPase Rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70:715-728.
- 561 35. Feliciano WD, Yoshida S, Straight SW, Swanson JA. 2011. Coordination of the Rab5 cycle on
 562 macropinosomes. Traffic 12:1911-1922.
- 563 36. Egami Y, Taguchi T, Maekawa M, Arai H, Araki N. 2014. Small GTPases and phosphoinositides
 564 in the regulatory mechanisms of macropinosome formation and maturation. Front Physiol 5:374.
- Selinka HC, Wolde A, Pasch A, Klingel K, Schnorr JJ, Küpper JH, Lindberg AM, Kandolf R. 2002.
 Comparative analysis of two coxsackievirus B3 strains: putative influence of virus-receptor
 interactions on pathogenesis. J Med Virol 67:224-233.
- Israelsson S, Sävneby A, Ekström JO, Jonsson N, Edman K, Lindberg AM. 2014. Improved
 replication efficiency of echovirus 5 after transfection of colon cancer cells using an authentic 5'
 RNA genome end methodology. Invest New Drugs 32:1063-1070.
- 39. Abraham G, Colonno RJ. 1984. Many rhinovirus serotypes share the same cellular receptor. J
 572 Virol 51:340-345.
- 40. Myllynen M, Kazmertsuk A, Marjomäki V. 2016. A novel open and infectious form of echovirus 1.
 J Virol 90:6759-6770.
- 575 41. Turkki P, Laajala M, Stark M, Vandesande H, Sallinen-Dal Maso H, Shroff S, Sävneby A, Galitska
 576 G, Lindberg AM, Marjomäki V. 2019. Slow infection due to lowering the amount of intact versus
 577 empty particles is a characteristic feature of coxsackievirus B5 dictated by the structural proteins.
 578 J Virol 93.
- 579 42. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden
 580 C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona
 581 A. 2012. Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676-682.
- 43. Manders EMM, Verbeek FJ, Aten JA. 1993. Measurement of co- localization of objects in dualcolour confocal images. J Microsc 169:375-382.
- 44. Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S. 2004. Automatic and
 quantitative measurement of protein-protein colocalization in live cells. Biophys J 86:3993-4003.

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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 µm. (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 (± 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 (C₁) value corresponds to a low amount of intracellular viral RNA. 598 Results are presented as mean values of 3 replicates (± standard deviation [SD]).

599

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

603

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

609

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

614

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

622

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

of the virus. The presence of capsid indicates viral replication. Red, viral capsid (DAKO); blue, nuclei
(DAPI). Scale bar is 20 µm.

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

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

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627

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

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

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647

654 FIG. 11. The effect of Rab5 on E30B infection. RD cells were transfected with a wild type (pEGFP-Rab5-WT), a dominant-negative (pEGFP-Rab5-S34N) or a constitutively active (pEGFP-Rab5-Q79L) 655 656 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 (± 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.





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I. Ó

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Mock

E30B (6 h p.i.)



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E30B

CVB5

FcRnsi

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DAKO

Merged

Mock

No drug

20 µm

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Cell viability %

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EEA1



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Transferri
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30 min. + CVA9

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