

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33

Title: The uncoating of EV71 in mature late endosomes requires CD-M6PR

Seii OHKA^{1,2,*}, Soon Hao TAN³, Eri ISHIYAMA⁴, Katsutoshi OGASAWARA⁴, Tomohito HANASAKA⁴, Kinji ISHIDA⁴, Kyoji HAGIWARA¹, Chia-Chyi LIU⁵, Pele Choi-Sing CHONG^{5,6}, Ken-ichi HANAOKI^{4,7}, Giampietro SCHIAVO^{8,9}

- 1. Neurovirology Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
- 2. Present address: Addictive Substance Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
- 3. Department of Pathology, University of Malaya, Kuala Lumpur, Malaysia
- 4. Technical Support Center for Life Science Research, Iwate Medical University, Iwate, Japan
- 5. National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Zhunan Town, Miaoli County, Taiwan
- 6. Present address: BravoVax Co., Ltd, Wuhan, China
- 7. Present address: Division of Experimental Animal Research, National Institute of Infectious Diseases, Tokyo, Japan
- 8. Queen Square Institute of Neurology, University College London, London, United Kingdom
- 9. UK Dementia Research Institute at UCL, London, United Kingdom

Running title: M6PR's roles in EV71 uncoating

Document totals:	Word count for Summary statement:	21
	Word count for Abstract:	152
	Word count for main text:	7,604

*Corresponding author:
Seii OHKA
Addictive Substance Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6, Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan
Phone: +81-3-5316-2390 Fax: +81-3-5316-2390
E-mail: ohka-si@igakuken.or.jp

34 **Keywords** EV71, late endosomes, mannose 6-phosphate receptor, uncoating, SCARB2

35

36

37

Summary Statement

38 In human rhabdomyosarcoma cells overexpressing human scavenger receptor B2, EV71

39 uncoating takes place in mature late endosomes and relies on CD-M6PR.

40

41

42

Abstract

43

44 Enterovirus 71 (EV71) is one of the causative agents of hand-foot-and-mouth disease, which

45 in some circumstances could lead to severe neurological diseases. Despite of its importance for

46 human health, little is known about the early stages of EV71 infection. EV71 starts uncoating with

47 its receptor, human scavenger receptor B2 (hSCARB2), at low pH. We show that EV71 was not

48 targeted to lysosomes in human rhabdomyosarcoma cells overexpressing hSCARB2 and that the

49 autophagic pathway is not essential for EV71 productive uncoating. Instead, EV71 was efficiently

50 uncoated 30 minutes after infection in late endosomes (LEs) containing hSCARB2, mannose-6-

51 phosphate receptor (M6PR), RAB9, bis(monoacylglycero)phosphate and lysosomal associated

52 membrane protein 2 (LAMP2). Furthering the notion that mature LEs are crucial for EV71

53 uncoating, cation-dependent (CD)-M6PR knockdown impairs EV71 infection. Since hSCARB2

54 interacts with cation-independent (CI)-M6PR through M6P-binding sites and CD-M6PR also harbor

55 a M6P-binding site, CD-M6PR is likely to play important roles in EV71 uncoating in LEs.

56

Introduction

Enterovirus 71 (EV71) is a causative agent of hand-foot-and-mouth disease (HFMD) in young children and infants ([Hagiwara et al., 1978](#), [Blomberg et al., 1974](#)), yet infection with this virus occasionally leads to severe neurological diseases, such as brainstem encephalitis and acute flaccid paralysis ([McMinn, 2002](#)). EV71 is classified into *Enterovirus* within the family *Picornaviridae* ([Racaniello, 2007](#)), which contains positive-sense single-stranded RNA surrounded by an icosahedral capsid proteins. Sixty copies of the four structural proteins VP1, VP2, VP3, and VP4 assemble to form the capsid ([Plevka et al., 2012](#), [Rossmann and Johnson, 1989](#), [Wang et al., 2012](#)). Functional EV71 receptor, human scavenger receptor class B, member 2 (hSCARB2, also known as lysosomal integral membrane protein II or CD36b like-2) ([Yamayoshi et al., 2009](#)), interacts with residues located within an indentation of the viral capsid, called the canyon ([Dang et al., 2014](#)). After the binding of hSCARB2 to the EV71 capsid, the virus particle releases viral genomic RNA using VP1 and VP4 under acidic conditions ([Yamayoshi et al., 2013](#), [Dang et al., 2014](#)).

hSCARB2, a CD36 family member containing two transmembrane domains ([Vega et al., 1991](#)), is an intrinsic receptor for β -glucocerebrosidase (β -GC) ([Eskelinen et al., 2003](#)). It transports β -GC from the endoplasmic reticulum (ER) to lysosomes ([Blanz et al., 2010](#), [Reczek et al., 2007](#)), and is required for lysosomal integrity ([Eskelinen et al., 2003](#)). In primate cells, lysosome sorting of β -GC by hSCARB2 is aided by cation-independent (CI)-mannose-6-phosphate (M6P) receptor (M6PR) ([Zhao et al., 2014](#)), which binds to a M6P group linked to amino acid 325 of hSCARB2; however, cation-dependent (CD)-M6PR may also contribute to the lysosome sorting of β -GC by hSCARB2 ([Zhao et al., 2014](#)). β -GC and EV71 bind overlapping regions of the intraluminal domain of hSCARB2: β -GC interacts with the 145-222 portion of hSCARB2 (in particular 150-167) ([Zachos et al., 2012](#), [Reczek et al., 2007](#)), whereas amino acids 142-204 (and in particular 144-151) are crucial

81 for EV71 binding ([Chen et al., 2012](#), [Yamayoshi and Koike, 2011](#)).

82 It has been reported that small interfering RNAs (siRNAs) against clathrin heavy chain
83 inhibit EV71 infection, suggesting that EV71 exploits a clathrin-dependent pathway for its entry into
84 target cells ([Lin et al., 2012](#), [Hussain et al., 2011](#)). After internalization, EV71 is delivered to early
85 endosomes ([Yamayoshi et al., 2013](#)), however, the precise mechanism for EV71 uncoating, such as
86 its timing and localization, has not been clarified. In the endocytic pathway, early endosomes
87 mature to late endosomes (LEs), which then fuse with lysosomes containing lysosomal associated
88 membrane protein (LAMP)-1 or 2. Progression along the endosomal route is regulated by specific
89 RAB GTPases, which serve as specific markers of these organelles. RAB5 regulates the early steps
90 in the endocytic pathway, participating to clathrin coated vesicle uncoating and formation of early
91 endosomes ([Semerdjieva et al., 2008](#), [Pfeffer, 2017](#)), whilst RAB7 regulates the transition between
92 early endosomes and LEs ([Wandinger-Ness and Zerial, 2014](#)), and RAB9 functions in LEs and the
93 trans-Golgi network ([Pfeffer, 2017](#)).

94 LAMP-1 and LAMP-2 are lysosomal membrane proteins, which share common functions
95 in vivo. Beyond their roles in maintaining the structural integrity of lysosomes, both LAMPs are
96 involved in the regulation of autophagic vacuoles and unesterified cholesterol in embryonic
97 fibroblasts ([Eskelinen, 2006](#)). In previous studies, SCARB2 was found to co-localize with LAMP-
98 2 in primate cells ([Zachos et al., 2012](#)).

99 Here, we report that EV71 is efficiently uncoated 30 minutes after infection (m.a.i.) in
100 mature LEs. Cation-dependent (CD)-M6PR was found to be essential for EV71 replication by
101 siRNA knockdown, suggesting that CD-M6PR may play an important role at early stages of EV71
102 infection.

103

Results

Analysis of EV71 uncoating kinetics

EV71 is uncoated upon binding to hSCARB2 at low pH ([Yamayoshi et al., 2013](#)). However, it is unclear where and when uncoating occurs in cells. To clarify when EV71 starts uncoating, we used light-sensitive EV71, which can be inactivated after irradiation at 450 nm. Human rhabdomyosarcoma RD cells overexpressing hSCARB2 (RD-hSCARB2), were infected with light-sensitive EV71 at a multiplicity of infection (MOI) of 10 and cells were either kept in the dark or irradiated at the indicated time after infection to inactivate intact virus. Seven hours after infection, cells were harvested and the viral titers were determined (Fig. 1A). In samples irradiated at either 5 or 10 m.a.i., virus titers decreased by 3 logs as compared to samples kept in the dark (Fig. 1A and 1B). In contrast, the viral titer of the sample irradiated at 20 m.a.i. was found to increase as compared to that in the 10 m.a.i. sample, and a further increase of viral titer was observed at 40 m.a.i. (Fig. 1A and 1B). The viral titer was found to plateau at 60 m.a.i., suggesting that the uncoating of EV71 is likely to start after 10 m.a.i. and that all the productive viral particles were uncoated before 40 m.a.i. The experiments were repeated three times and similar results were observed.

Next, we combined in situ hybridization and immunofluorescence to visualize the viral RNA as well as the viral capsid. RD-hSCARB2 cells were infected with purified EV71 at an MOI of 25 and cells were fixed at specific times after infection, followed by immunofluorescence and in situ hybridization (Fig. 1C). The number of EV71 genomic RNA puncta per cell were analyzed (Fig. 1D); we found 1.4, 0.9 and 3.2 for 0 m.a.i., 2.8, 4.0 and 1.3 for 10 m.a.i., 4.2, 13.5 and 7.0 for 20 m.a.i., 46.0, 30.8 and 44.9 for 30 m.a.i., 1.6 and 2.0 for 30 m.a.i. with NH₄Cl. At 0 m.a.i., we observed negligible amounts of EV71 genomic RNA (1.8 puncta per cell), whereas we detected EV71 capsid on the cell surface. This result suggests that EV71 genomic RNA inside the capsid cannot be

128 detected by our method. At 10 m.a.i., we detected EV71 capsid, but we observed only 2.7 puncta
129 of EV71 genomic RNA per cell, indicating that EV71 was not uncoated efficiently at this time point.
130 At 20 m.a.i., we observed 8.2 puncta of EV71 genomic RNA per cell, whilst at 30 m.a.i., 40.6 puncta
131 of EV71 genomic RNA per cell were observed. The amount of EV71 genomic RNA significantly
132 increased around five times at 30 m.a.i. compared to that at 20 m.a.i. Furthermore, EV71 RNA and
133 capsid double-labelled vesicles, and EV71 RNA abutting capsid-positive structures underwent
134 clustering at 30 m.a.i, suggesting that uncoating of EV71 robustly occurs at this time point (n = 2
135 independent experiments).

136 To confirm whether acidification is required for uncoating, cells were treated with NH₄Cl,
137 which blocks acidification of the lumen of endocytic organelles, and the presence of EV71 genomic
138 RNA was then assessed. RD-hSCARB2 cells were pretreated with or without 40 mM NH₄Cl for 30
139 minutes followed by addition of EV71 at an MOI of 25. As shown in Fig. 1C, we observed only 1.8
140 puncta of EV71 genomic RNA per cell even at 30 m.a.i. upon incubation with NH₄Cl, demonstrating
141 that an acidic pH is absolutely required for EV71 uncoating. These experiments were repeated three
142 times. Our results confirm previous finding that EV71 uncoating is triggered by low pH ([Yamayoshi
143 et al., 2013](#), [Chen et al., 2012](#)), and suggest that this immunofluorescence approach is a reliable
144 approach for the detection of viral uncoating.

145

146 **EV71 localizes to LEs during uncoating**

147 It has been hypothesized that EV71 requires hSCARB2 for its uncoating ([Yamayoshi et al.,
148 2013](#)). To begin with, we examined whether EV71 traffics with hSCARB2 during the uncoating
149 period using immunofluorescence (Fig. 2). RD-hSCARB2 cells were infected with EV71 at an MOI
150 of 25, and fixed at 5, 10, 20, 30, and 40 m.a.i. followed by immunofluorescence. We detected 129,
151 111, 102, 123, and 102 EV71-positive puncta at 5, 10, 20, 30, and 40 m.a.i., respectively. As shown

152 in Fig. 2A and 2E (Fig. 2A for 40 m.a.i.; immunofluorescence at 5, 10, 20, 30 m.a.i. not shown; Fig.
153 2E, co-localization kinetics), EV71 displays a high level of co-localization with hSCARB2 ranging
154 from 20 to 30% until 40 m.a.i., suggesting that EV71 is sorted together with hSCARB2 in the
155 uncoating period. Next, we examined the intracellular localization of EV71 during this period. As
156 stated in the Materials and Methods, anti-early endosome antigen 1 (EEA1) antibodies were used to
157 identify early endosomes (EE), an anti-CI-M6PR/IGF2R antibody was used for LEs and anti-LAMP1
158 antibodies were used as lysosome marker. We analyzed a large number of EV71-positive puncta:
159 60, 92, and 81 at 5 m.a.i., 102, 112, and 100 at 10 m.a.i., 121, 112, and 93 at 20 m.a.i., 103, 101, and
160 101 at 30 m.a.i., 100, 101, and 103 at 40 m.a.i., for EEA1, CI-M6PR, and LAMP1, respectively.
161 Interestingly, EV71 were found to co-localize with CI-M6PR in around 20% of the EV71-positive
162 organelles from 10 to 30 m.a.i. (Fig. 2C for 30 m.a.i.; immunofluorescence data at 5, 10, 20, 40 m.a.i.
163 not shown; Fig. 2E, co-localization kinetics), whereas only 10% of EV71 co-localized with EEA1 or
164 LAMP1 puncta at this time point (Fig. 2B and 2D for 30 m.a.i.; immunofluorescence at 5, 10, 20, 40
165 m.a.i. not shown; Fig. 2E, co-localization kinetics; n=3 independent experiments). We confirmed
166 that all EV71 puncta positive for the above markers also co-localize with hSCARB2.

167 As a control, we checked the intracellular localization of epidermal growth factor (EGF),
168 which is known to traffic to LAMP1-positive LE/lysosomes 30 minutes after internalization (Fig. 3)
169 ([Chin et al., 2020](#)). As expected, EGF co-localized with LAMP1 in 48.5% of the organelles,
170 whereas it co-distributes with CI-M6PR only in 24.0% of the puncta (50 EGF + CI-M6PR double-
171 labelled vesicles in 253 EGF-positive puncta; 123 EGF + LAMP1 double-labelled vesicles in 261
172 EGF-positive puncta; 9 EGF + SCARB2 double-labelled vesicles in 117 EGF-positive puncta).
173 These results suggest that EGF is targeted to lysosomes 30 minutes after internalization in our
174 experimental conditions (n=2 independent experiments).

175 To confirm EV71 localization during uncoating, we tested the co-distribution of EV71 with
176 the endosomal markers RAB5, RAB7, and RAB9. RAB5 mainly localizes on early endosomes,
177 whereas RAB7 and RAB9 localizes on specific sub-classes of LEs. RD-hSCARB2 cells were
178 infected with EV71 at an MOI of 25, and fixed at 5, 10, 20, 30, and 40 m.a.i. followed by
179 immunofluorescence. We analyzed the following number of EV71-positive puncta; 153, 135, and
180 128 at 5 m.a.i., 182, 143, and 150 at 10 m.a.i., 145, 154, and 137 at 20 m.a.i., 133, 174, and 150 at 30
181 m.a.i., 35, 117, and 102 at 40 m.a.i. for RAB5, RAB7, and RAB9, respectively. EV71 shows an
182 extensively colocalization with RAB9 from 10 m.a.i. in over 20% of the organelles (Fig. 4C and 4D),
183 whereas the co-distribution with RAB5 or RAB7 occurs in less than 20% of the cases during this
184 period (Fig. 4A, 4B, and 4D; data at 5, 10, 20, 40 m.a.i. are not shown; n=3 independent experiments).
185 These results indicate that EV71 enters RAB9-positive LE during the uncoating period.

186 The data shown in Fig. 2 suggest that EV71 is not targeted to LAMP1-positive
187 endolysosomes. Since it has been reported that hSCARB2 co-localized with LAMP2 ([Reczek et al.,
188 2007](#)), EV71 may be targeted to LAMP2-positive organelles during uncoating. To investigate
189 whether this is the case, RD-hSCARB2 cells were infected with EV71 at an MOI of 25, and fixed at
190 30 and 40 m.a.i., followed by immunofluorescence (Fig. S1A and C). Around 20% of EV71 were
191 found to co-localize with LAMP2 at 30 m.a.i., whereas the co-localization decreases around 5% at
192 40 m.a.i. The experiments were repeated three times and the same trend was observed. These
193 results suggest that the organelles containing EV71 are positive for LAMP2 at 30 m.a.i., but LAMP2
194 is sorted away before 40 m.a.i. Altogether, our findings demonstrate that EV71 is targeted to
195 endocytic organelles containing LAMP2, RAB9, and CI-M6PR at 30 m.a.i. prior to uncoating.

196

197 **Uncoating of EV71 does not require LAMP2**

198 It has been previously reported that a specific pool of autophagosomes contains LAMP2,

199 RAB9, and M6PR ([Nishida et al., 2009](#)) and that 3-methyladenine (3-MA), which inhibits formation
200 of amphisomes ([Nishida et al., 2009](#), [Juenemann and Reits, 2012](#), [Sun et al., 2015](#)), halts immature
201 LEs to gain LAMP2. To examine whether blocking autophagy affects the co-localization of EV71
202 with LAMP2, RAB9, and M6PR at 30 and 40 m.a.i., cells were treated with 3-MA and organelles
203 containing EV71 were analyzed by immunofluorescence and quantified (Fig. S1B and C). RD-
204 hSCARB2 cells were pretreated with or without 5 mM 3-MA for 30 minutes followed by addition of
205 EV71 at an MOI of 25. We analyzed the following numbers of EV71-positive puncta; 106, 130, and
206 140 at 30 m.a.i. without 3-MA, 129, 120, and 202 at 30 m.a.i. with 3-MA, 132, 181, and 125 at 40
207 m.a.i. without 3-MA, 103, 145, and 109 at 40 m.a.i. with 3-MA, for LAMP2, RAB9, and CI-M6PR,
208 respectively. In the presence of 3-MA, EV71 was not targeted to an intracellular compartment
209 positive for LAMP2 even at 40 m.a.i. Interestingly, 3-MA did not affect the ability of EV71 to reach
210 organelles containing RAB9 or CI-M6PR at 30 and 40 m.a.i. The experiments were repeated three
211 times with similar results. These findings suggest that 3-MA blocks the targeting of EV71 to
212 LAMP2-positive organelles, yet does not alter its co-localization with RAB9 or CI-M6PR.

213 To investigate whether LAMP2 is necessary for EV71 uncoating, the effect of 3-MA on light-
214 sensitive EV71 was examined (Fig. S2A). RD-hSCARB2 cells were infected with light-sensitive
215 EV71 at an MOI of 0.004 in the presence or absence of 5 mM 3-MA. Cells were either kept in the
216 dark or irradiated at 450 nm at 10 or 40 m.a.i. to inactivate the intact virus. After irradiation, in
217 selected samples, 3-MA-containing medium was replaced with fresh medium to assess the inhibitory
218 effect this compound on viral replication. Twenty-three hours after infection, cells were harvested
219 and the viral titers determined.

220 In the light-treated samples, viral uncoating was blocked by irradiation, hence the effect of
221 viral uncoating on the viral replication was restricted to the pre-irradiation period. When cells were
222 irradiated at 10 m.a.i., the virus titer decreased by around 5 logs compared to cells kept in the dark in

223 the absence of 3-MA, indicating that this genetically-engineered virus was indeed very sensitive to
224 light. First, we investigated the effect of 3-MA on the productive uncoating of the virus. Light
225 irradiation inhibits additional viral uncoating to enable solely observing the productive uncoating
226 before light irradiation. With light irradiation, 3-MA significantly decreased viral titer in 3-MA (+)
227 to (-) and in 3-MA (+) ($P = 0.0118$ and $P = 0.0057$, respectively; unpaired t -test) compared to 3-MA
228 (-). These results indicate that 3-MA inhibited viral productive uncoating before light irradiation,
229 although the virus titer decreased only less than one order of magnitude. Next, we investigated the
230 effect of 3-MA on the replication of the virus. Treatment with 3-MA during viral replication after
231 light irradiation (3-MA (+)) reveals the effect of autophagy on the viral replication process. 3-MA
232 did not decrease viral titer in 3-MA (+) compared to 3-MA (+) to (-) ($P > 0.05$ by unpaired t -test),
233 suggesting that 3-MA did not have a significant effect on the viral replication after light irradiation.
234 No significant differences were found in samples kept in the dark, indicating the viral uncoating
235 progressed after 40 m.a.i. in absence of illumination. The experiments were repeated three times
236 and similar results were observed. These findings suggest that 3-MA has only a limited effect on
237 EV71 productive uncoating, and that this process does not require transit through LAMP2-positive
238 organelles.

239 To further explore whether EV71 uncoating fully proceed in the presence of 3-MA, we
240 analyzed the influence of 3-MA on viral uncoating by combined in situ hybridization and
241 immunofluorescence (Fig. S2B). RD-hSCARB2 cells were infected with EV71 at an MOI of 25
242 with or without 3-MA, prior to fixing at specific times after infection. At 0 m.a.i., EV71 RNA was
243 not detected, in agreement to the results shown in Fig. 1C, whilst at 30 m.a.i., viral RNA was detected
244 even in the presence of 3-MA. These results confirm that EV71 undergoes uncoating in the presence
245 of 3-MA in a process that does not require LAMP2. To further confirm EV71 undergoes uncoating
246 in LE in the presence of 3-MA, co-localization of EV71 RNA and CI-M6PR was observed (Fig. S2C).

247 At 20 m.a.i., the numbers of EV71 RNA + CI-M6PR double labelled puncta in the absence or presence
248 of 3-MA were 19 and 18, respectively, and the numbers of EV71 RNA labelled puncta in the absence
249 or presence of 3-MA was 106 and 64, respectively. At 30 m.a.i., the EV71 RNA + CI-M6PR double-
250 labelled puncta in the absence or presence of 3-MA were 15 and 48, respectively, whereas the EV71
251 RNA-labelled puncta in the absence or presence of 3-MA were 167 and 200. Co-localization rate
252 of EV71 RNA and CI-M6PR was significantly higher in the presence of 3-MA compared to that
253 observed in the absence of the compound at 30 m.a.i. A similar trend was seen at 20 m.a.i.

254 In summary, these results suggest that EV71 undergoes uncoating in LEs containing CI-
255 M6PR even in the presence of 3-MA, and this inhibitor boosts the rate of EV71 uncoating in LEs
256 containing CI-M6PR.

257

258 **Uncoating of EV71 occurs in mature LEs containing intraluminal vesicles**

259 EV71 uncoating starts between 20 m.a.i., when uncoating is negligible, and 30 m.a.i., a time-
260 point in which robust uncoating is observed, yet we lack key information on the nature of these
261 organelles. To further investigate the intracellular trafficking of EV71, we performed transmission
262 electron microscopy (TEM). RD-hSCARB2 cells were infected with purified EV71 at an MOI of
263 25, fixed at 20 and 30 m.a.i., and then analyzed by TEM (Fig. 5A). At 20 m.a.i., viral particles were
264 surrounded by a single membrane bilayer in organelles resembling Les, but lacking intraluminal
265 vesicles (ILVs). At 30 m.a.i., the viral particles reach LEs containing in some instances ILVs (n=3).
266 These results imply that EV71 is present in the lumen of LEs during its uncoating.

267 To confirm this conclusion, samples were examined by immunoelectron microscopy (Fig.
268 5B). At both 20 and 30 m.a.i., gold particles labelling EV71 were found in the lumen of single
269 membrane organelles, likely to be LEs. At 30 m.a.i., gold particles accumulated in single membrane
270 organelles containing ILVs. These results suggest that EV71 accumulates in LE at 20 and 30 m.a.i.

271 and that during the uncoating period (30 m.a.i.), EV71 specifically localizes at LEs containing ILVs.

272 It has been reported that ILVs accumulate in LEs following maturation of this endosomal
273 compartment ([Nour and Modis, 2014](#)). ILVs in mature LEs are enriched in M6PRs and
274 bis(monoacylglycero)phosphate (BMP) ([Nour and Modis, 2014](#)). Based on these considerations, it
275 is plausible that the organelles containing EV71 at 20 m.a.i. are immature LEs, which undergo
276 maturation at 30 m.a.i. acquiring ILVs enriched in BMP. To examine whether the BMP content
277 differs between the vesicles containing EV71 at 20 m.a.i. and those at 30 m.a.i., RD-hSCARB2 cells
278 were infected with EV71 at an MOI of 25, fixed at 20 and 30 m.a.i. and analyzed by
279 immunofluorescence (Fig. 6). We analyzed the following numbers of EV71-positive puncta: 204,
280 13, 35 at 20 m.a.i. and 317, 83 at 30 m.a.i.; EV71 + BMP double-labelled puncta were 19, 1, 1 at 20
281 m.a.i. and 144, 34 at 30 m.a.i., respectively. At 20 m.a.i., 6.6% of EV71 co-localized with BMP,
282 whereas the percentage of EV71 co-localized with BMP increased to 43.1% at 30 m.a.i. (n=2
283 independent experiments), suggesting that LE maturation is important for efficient uncoating of the
284 virus.

285

286 **CD-M6PR is required for EV71 replication**

287 To investigate whether RABs or M6PRs are essential for EV71 trafficking and replication,
288 we analyzed viral replication in cells in which specific components of the endocytic pathway were
289 downregulated by siRNA. RD cells targeted by siRNA knockdown were infected with EV71-GFP
290 at an MOI of 0.5, fixed after 31 hours from infection prior to fluorescent imaging to assess the
291 percentage of GFP-positive cells in the culture. Protein expression levels in RD cells treated with
292 siRNA were examined by western blotting (Fig. 7A). As shown in Fig. 7B, CD-M6PR siRNA
293 knockdown showed significant inhibition of EV71 replication compared to cells treated with control
294 siRNA ($P = 0.049$), whereas RABs or clathrin heavy chain downregulation did not have any effect

295 (n=3 independent experiments). These results suggest that RAB5, RAB7, and RAB9 are not
296 essential for EV71 replication, whereas CD-M6PR is required for this process, including virus
297 uncoating.

298

Discussion

299

300

301 Although EV71 co-localized with LAMP2, RAB9, and M6PR during its uncoating period,
302 our results suggest that LAMP2 and RAB9 are not essential for EV71 replication. Based on these
303 findings, amphisomes containing LAMP2, RAB9, and M6PR are not likely to be required for EV71
304 uncoating. Indeed, we could not find EV71 in organelles having double limiting membranes
305 similar to those described for autophagosomes or amphisomes. Thus, EV71 is likely to localize to
306 LEs, and not to amphisomes during the uncoating period (30 to 40 m.a.i.). At 40 m.a.i., it was
307 difficult to detect virus particles by immunoelectron microscopy. Accordingly, the number of viral
308 antigens observed by immunofluorescence was highly reduced at 40 m.a.i. compared to that at 30
309 m.a.i. or earlier timepoints (data not shown). From these results, the compartments containing
310 EV71 are predicted to undergo fusion with degradative organelles before or at 40 m.a.i. Indeed,
311 RAB9-positive organelles have been reported to fuse with compartments containing RAB7
312 ([Stenmark, 2009](#)), a process that might lead to the degradation of EV71 present in their lumen.
313 ([Kirkegaard, 2013](#)). Furthermore, since hSCARB2 and LAMP2 target their binding proteins to
314 lysosomal degradation ([Blanz et al., 2010](#), [Reczek et al., 2007](#), [Cuervo and Dice, 1996](#), [Ebato et al.,](#)
315 [2008](#)), it is likely that both hSCARB2 and LAMP2 direct EV71 to the degradative pathway. This
316 may explain why we observe an apparent increase of EV71 RNA upon treatment with 3-MA, when
317 organelles containing EV71 does not acquire LAMP2 (Fig. S2C). In the presence of 3-MA, 24%
318 of the viral RNA co-localized with M6PR, in stark contrast to the 9.0% observed in the absence of
319 3-MA at 30 m.a.i. However, we cannot rule out the possibility that overexpression of hSCARB2
320 may affect the dynamics of the virus in these cells and as such, influence some of our findings.

321 Since EV71 starts replication in the cytoplasm by IRES-mediated initiation of translation
322 ([Thompson and Sarnow, 2003](#)), it is likely that the entry of genomic viral RNA in the cytoplasm

323 leads to productive infection. On the other hand, the viral RNA present in the lumen of LEs would
324 be exposed to a degradative environment, leading to abortive infection (Fig. S1). In the case the
325 uncoating of the virus occurs on ILVs in LEs, viral RNA is released into LE lumen, whereas when
326 the uncoating occurs on LE membrane, viral RNA is released into cytoplasm ([Dang et al., 2014](#)).
327 As shown in Fig. S2C, we observed more EV71 RNA in the presence of 3-MA than in the absence
328 of 3-MA, whereas 3-MA inhibited virus productive uncoating (Fig. S2A). These results suggest
329 that some of the EV71 uncoating occur on ILVs in LEs and 3-MA inhibits uncoated EV71 RNA to
330 leave LEs at 30 m.a.i. Thus, it is likely that 3-MA increases abortive infection of EV71.
331 According to a previous report ([Lee et al., 2014](#)), 3-MA reduced the replication and pathogenesis of
332 EV71 (strain 4643) in a suckling mouse model. In 16HBE cells, 10 mM 3-MA inhibited the
333 replication of EV71 (sub-genotype C4, GenBank: EU812515.1) ([Song et al., 2018](#)). Our strategy
334 utilizing RD-hSCARB2 cells and light sensitive virus allowed us to analyze viral uncoating and
335 replication separately. As a result, we found that 5 mM 3-MA did not affect the productive
336 uncoating of EV71 and did not have a significant effect on viral replication (Fig. S2A).

337 The pH of the immature LEs is around pH 6.0, whereas mature LEs enriched in BMP-
338 containing ILVs is pH 5.5 or lower ([Nour and Modis, 2014](#)). EV71 bound to hSCARB2
339 significantly starts uncoating at pH 6.0 or below in vitro ([Yamayoshi et al., 2013](#)). At pH 6.5,
340 2.6% of the virus was uncoated after 1 h, whereas this percentage raises to 5.0% and 6.1% at pH 6.0
341 or pH 5.5, respectively. From these results, only cellular compartments with a pH of 6.0 or lower
342 would be compatible with viral uncoating. However, in the case of poliovirus (PV), which
343 requires solely its receptor for uncoating, about 90% of the virus particles lose their coat 1 hour
344 after addition of its receptor ([Arita et al., 1998](#)). Thus, the efficiency for uncoating of EV71 bound
345 to hSCARB2 at pH 6.0 is likely to be much lower than that of PV. This difference might imply
346 that other factors assist EV71 uncoating besides hSCARB2 and the low pH.

347 Our results suggest that CD-M6PR is essential for EV71 replication, including uncoating.
348 CD-M6PR is enriched in ILVs ([Dikic, 2006](#)). Both CI-M6PR and CD-M6PR have M6P-binding
349 sites, and CI-M6PR interacts with hSCARB2 holding β -GC via M6P in cis ([Zhao et al., 2014](#)).
350 Since CD-M6PR has a short extracellular domain and hSCARB2 is modified with M6P at the distal
351 site from the membrane, CD-M6PR might interact with M6P on hSCARB2 in trans. The
352 hypothesized interaction between CD-M6PR and hSCARB2 in trans would facilitate the binding
353 between EV71 with another hSCARB2 molecule. It is possible that the action of multiple
354 hSCARB2 molecules localized on both LEs and the ILVs would enhance the uncoating kinetics of
355 EV71. However, future work will be necessary to validate this hypothesis. Since knockdown of
356 M6PR may influence the distribution of SCARB2, we cannot exclude the possibility that M6PR
357 interferes indirectly with the uncoating of the virus.

358 Efficient uncoating of EV71 occurs at mature LEs containing ILVs. Both immature and
359 mature LEs have a pH low enough for EV71 uncoating; however, EV71 is not uncoated efficiently
360 in immature LEs. A possible reason for this might reside in the structural difference between
361 immature and mature LEs. In immature LEs, which harbour fewer ILVs, EV71 would be mainly
362 localized mainly on the delimiting LE membrane, since uncoating of the virus occurs solely on
363 membranes. In contrast, in mature LEs, both EV71 and hSCARB2 would localize on LE and ILV
364 membranes, thus increasing the potential for uncoating. An alternative, yet not exclusive
365 possibility is linked to the pH dependent interaction of hSCARB2 with β -GC. Previous in vitro
366 studies demonstrated that hSCARB2 binds β -GC at pH 6.5, whereas it does not at pH 5.5 ([Zhao et](#)
367 [al., 2014](#)). Interestingly, the binding site(s) of hSCARB2 for β -GC (amino acids 145-222 ([Zachos](#)
368 [et al., 2012](#)) or 150–167 ([Reczek et al., 2007](#))) overlaps with the binding site(s) for EV71 (amino
369 acids 142-204 ([Yamayoshi and Koike, 2011](#)) or 144–151 ([Chen et al., 2012](#))), and exogenous β -GC
370 has been found to interfere with EV71 binding to hSCARB2 on the cell surface ([Nakata et al.,](#)

371 [2017](#)). This implies that release of β -GC from hSCARB2 at pH 5.5 may enhance the interaction

372 between EV71 and hSCARB2, thus boosting EV71 uncoating.

373

Materials and Methods

374

375

376 Cells

377 RD cells and Vero cells were cultured in Dulbecco's modified Eagle's medium (Nissui)
378 supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin (Life Technologies)
379 (5% FBS-DMEM). RD-hSCARB2 cells (generously gifted by Dr. K. Fujii) were cultured in 5%
380 FBS-DMEM supplemented with 4 µg/ml puromycin (Calbiochem). Cells had been tested for
381 contamination and mycoplasma infection.

382 Viruses

383 The EV71 strains SK-EV006/Malaysia/97 (SK-EV006; genogroup B) ([Shimizu et al., 1999](#)),
384 EV71/86-11316 NETH-86 (genogroup B) ([Nakajima et al., 2012](#)) and EV71-GFP (generously gifted
385 by Dr. M. Arita), which expresses GFP upon viral replication ([Yamayoshi et al., 2013](#)), were used in
386 this study. EV71/86-11316 NETH-86 was used for producing light-sensitive virus, and EV71-GFP
387 was used in siRNA knockdown experiment. Unless otherwise stated, we used purified EV71 SK-
388 EV006 as a representative strain of EV71. Experiments using recombinant DNA and pathogens
389 were approved by the Committee for Experiments using Recombinant DNA and Pathogens at the
390 Tokyo Metropolitan Institute of Medical Science (TMiMS).

391 Antibodies

392 We used the following primary antibodies for immunofluorescence and western blotting:
393 anti-SCARB2 goat antibodies (1:1250, AF1966, R&D Systems), anti-EEA1 rabbit antibodies (1:500,
394 ab2900, Abcam), anti-clathrin heavy chain rabbit antibodies (1:1,000, ab21679, Abcam), anti-RAB5
395 rabbit antibodies (1:1,000, ab18211, Abcam), anti-RAB7 rabbit monoclonal antibody [EPR7589]
396 (1:250 for immunofluorescence, 1:1,000 for western blotting, ab137029, Abcam), anti-RAb9 mouse
397 monoclonal antibody [EPR13272] (1:100 for immunofluorescence, 1:1,000 for western blotting,

398 ab179815, Abcam), anti-LAMP1 rabbit antibodies (1:1,000, ab24170, Abcam), purified anti-LBPA
399 mouse antibody (6C4) for BMP staining (1:500, Z-PLBPA, Echelon), anti-LAMP2 [EPR13272]
400 rabbit monoclonal antibody (1:100, ab37024, Abcam), anti-EV71 mouse monoclonal antibody N3
401 (1:9000, from G. Liu and P. C. Choi-Sing), anti-EV71 serum (1:1,000 for immunofluorescence, 1:200
402 for immunoelectron microscopy, kindly provided by H. Shimizu, NIID, Japan) ([Nagata et al., 2002](#)),
403 anti- β -actin AC74 monoclonal antibody (1:5,000, A5316, Sigma-Aldrich). For anti-M6PR
404 antibodies, we used anti-CI-M6PR/IGF2R rabbit monoclonal antibody [EPR6599] (1:250, ab124767,
405 Abcam), anti-CD-M6PR rabbit polyclonal antibodies (1:50, ABIN357957, Antibodies-online) and
406 chicken polyclonal antibodies (1:200, GW21444, Sigma) for immunofluorescence, and anti-CI-
407 M6PR/IGF2R rabbit antibody [EPR6599] (1:50,000, ab124767, Abcam), anti-CD-M6PR chicken
408 polyclonal antibodies (1:500, GW21444, Sigma) for western blotting. As secondary antibodies, we
409 used AlexaFluor488, 568, and 647 donkey, or goat anti-mouse, rabbit, chicken or goat IgG (H+L)
410 (1:1,000, Life Technologies), anti-IgG (H+L), 5 nm gold-conjugated goat anti-rabbit IgG (H+L), EM
411 (1:100, EM.GARHL5, BBI Solutions), anti-rabbit IgG HRP, and anti-mouse IgG HRP (1:100,000,
412 Jackson ImmunoResearch).

413 **Virus purification**

414 RD-hSCARB2 cells were infected with EV71 SK-EV006/Malaysia/97 strain at an MOI of
415 5. Cells and media were frozen 18 hours post-infection. After thawing, cell debris was removed by
416 centrifugation in a NA-4HS rotor (TOMY) at 10,000 rpm for 20 minutes at 4°C. The supernatant was
417 layered onto a 1.25 g/ml and 1.48 g/ml CsCl discontinuous step gradient in phosphate-buffered saline
418 [PBS (-)] (per liter, 8.00 g NaCl, 1.15 g Na₂HPO₄, 0.20 g KCl, 0.20 g KH₂PO₄ [pH 7.4]). Native
419 virions (F-particles) were collected from a fraction between the 1.25 g/ml and 1.48 g/ml CsCl. The
420 F-particles were applied onto PD-10 or NAP5 column and eluted with PBS (-). After fractionation
421 (0.2 ml/fraction), the fractions that included the F-particles were collected.

422 **Light-sensitive EV71**

423 The experiments were performed in the dark unless stated otherwise. RD-hSCARB2 cells
424 were infected with EV71/86-11316 NETH-86 strain at an MOI of 0.1 in 10 ml of 5% FBS-DMEM
425 and incubated at 37°C for 1 hour. Upon removal of the supernatant, cells were washed with DMEM
426 and then 20 ml of 5% FBS-DMEM and 40 µg/ml neutral red were added. Around 24 hours after
427 infection, the infected cells were transferred at -80°C followed by freezing and thawing three times.
428 After a sonication for 30 minutes on ice, the virus solution was centrifuged and the supernatant was
429 recovered followed by titration in the dark or after light irradiation using TCID₅₀ in VERO cells.
430 RD-hSCARB2 was infected with the recovered virus similarly to the first stage infection. The virus
431 supernatant was recovered followed by titration using TCID₅₀ in VERO cells and used for
432 experiments. For light irradiation of the light-sensitive virus, light was shone 30 cm apart from the
433 plates by Grassy LeDio RS122 Fresh White LED light (Volxjapan) including 450 nm spectrum peak
434 for 30 minutes at room temperature. The temperature of the plates was strictly monitored to avoid
435 cell overheating. In experiments described in Fig. 1A and 1B, RD-hSCARB2 cells were infected
436 with light-sensitive EV71 at a MOI of 10 and cells were either kept in the dark or irradiated at the
437 indicated time after infection to inactivate intact virus. Experiments were performed in triplicate.
438 Seven hours after infection, cells were harvested and the viral titers were determined using TCID₅₀
439 in VERO cells. In the experiments shown Fig. S2A, RD-hSCARB2 cells were infected with light-
440 sensitive EV71 at an MOI of 0.004 in the presence or absence of 5 mM 3-MA. Cells were either
441 kept in the dark or irradiated with light to inactivate the intact virus at 10 or 40 m.a.i. After
442 irradiation, the medium containing 3-MA was replaced with fresh medium in selected samples to
443 assess the inhibitory effect of 3-MA on viral replication. These experiments were performed in
444 triplicate. Twenty-three hours after infection, cells were harvested and the viral titers determined
445 using TCID₅₀ in VERO cells.

446 **Immunofluorescence**

447 RD-SCARB2 cells and RD cells were seeded onto 16-well Lab-Tek Chamber Slide (Nunc).
448 One day after seeding, purified EV71 were added at an MOI of 25 at 4°C to allow viral attachment
449 to the cell surface without entry. In selected cases, cells were pretreated with or without 40 mM
450 NH₄Cl or 5 mM 3-MA for 30 minutes. Cells were then shifted to 37°C (designated as time 0),
451 washed with PBS (-) with or without NH₄Cl or 3-MA, fixed in PBS containing 4% paraformaldehyde
452 (PFA) for 15 minutes and washed four times with cold PBS before further processing. In Fig. 3, to
453 detect EGF localization, we added 2 µg/ml of biotinylated EGF bound to AlexaFluor555 streptavidin
454 (Invitrogen) in the medium. Fixed cells were incubated with PBS containing 0.05% saponin and
455 5% bovine serum albumin fraction V (Sigma) to permeabilize the membrane and block nonspecific
456 reactions. Samples were incubated overnight at 4°C with primary antibodies. After being washed
457 with PBS (-), samples were then incubated with the secondary antibodies for 90 minutes at room
458 temperature, and after another PBS (-) wash, mounted in Vectashield with DAPI Mounting Medium
459 (Vector Laboratories). Samples were imaged with a laser-scanning microscope (TCS SP2, Leica
460 Microsystems). Images were analyzed by Imaris (Zeiss).

461 **In situ immunofluorescence analysis**

462 Cells were pretreated with or without 40 mM NH₄Cl or 5 mM 3-MA for 30 minutes followed
463 by addition of purified EV71 at an MOI of 25 with or without NH₄Cl or 3-MA. Infected cells were
464 washed twice with PBS (-) with or without NH₄Cl or 3-MA followed by fixation with 4% PFA at
465 room temperature for 30 minutes. Fixed cells were washed with PBS (-) 3 times, dehydrated with
466 ethanol and transferred at -20°C. After rehydration, samples were treated with proteases and in situ
467 hybridization was performed using QuantiGene ViewRNA ISH (Thermo Fisher) according to
468 manufacturer's instructions followed by fluorescence analysis. EV71 sequence was from nucleotide
469 number 3008 to 3609 of SK-EV006/Malaysia/97 strain (GenBank: AB469182.1) ([Shimizu et al.](#),

470 [1999](#)).

471 **EV71 infection in knockdown cells**

472 RD cells were seeded in 8 well culture slides (BD Falcon) one day before transfection with
473 siRNAs. siRNAs, pre-designed or validated *Silencer* Select siRNAs were obtained from Life
474 Technologies. As a negative control, *Silencer* Negative Control No. 1 siRNA (Life Technologies)
475 was used. Transfection of siRNAs into RD cells was performed using Lipofectamine 3000
476 (Invitrogen, Carlsbad, CA), which was diluted in Opti-MEM and added to siRNA diluted in Opti-
477 MEM. After an incubation of 5 minutes at room temperature, the mixture was added to cells and
478 incubated at 37°C for 4 hours followed by medium exchange. Two days later, cells were infected
479 with EV71-GFP at an MOI of 0.5. Experiment were performed in triplicate for knockdown samples
480 and sextuplicate for siControl. In parallel, cells in other wells were fixed in PBS (-) containing 4%
481 PFA for 30 minutes followed by immunostaining of target proteins to check the knockdown efficiency.
482 Immunostaining showed knockdown efficiency consistent with the results obtaining using western
483 blotting. After 24 hours incubation at 37°C, infected cells were fixed in PBS containing 4% PFA
484 for 30 minutes and washed four times with PBS. The numbers of GFP-positive cells were counted
485 and the ratio against the numbers of DAPI-positive cells was analyzed. GFP positive cells were
486 observed by VS120 Virtual Slide Microscope (Olympus) and analyzed using Image J software
487 (National Institutes of Health, USA). To analyze protein expression by immunofluorescence, cells
488 were stained as described above and imaged with a laser-scanning microscope. To quantify protein
489 expression, samples were loaded onto 12% Mini-Protean TGX precast gels (Bio-Rad), followed by
490 western blotting with anti-RAB5, RAB7, RAB9, M6PRs, and clathrin heavy chain antibodies. RD
491 cells treated with siRNAs were detached with 0.02% EDTA-PBS and dissolved in 100 µl of
492 electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate,
493 0.1% bromophenol blue in H₂O) per 2×10^5 cells, followed by sonication and addition of 5% v/v of

494 2-mercaptoethanol. Samples were loaded onto precast gels mini-Protein TGX Gels 12% (BioRad),
495 followed by western blotting. Band intensities were analyzed using Image J.

496 **Electron microscopy**

497 For transmission electron microscopy, cells washed with PBS (-) were fixed in 2% (w/v)
498 PFA and 2.5% (v/v) glutaraldehyde in PBS (-) for 2.5 h. After osmication in 1% (w/v) osmium
499 tetroxide, specimens were dehydrated through a graded alcohol series, and embedded in Epon 812
500 (TAAB Laboratories). Ultrathin sections were cut on an Ultracut microtome (Leica EM-UC6) and
501 stained with uranyl acetate and lead citrate. Sections were observed under a transmission electron
502 microscope (Hitachi H-7650).

503 For immuno-electron microscopy, cells washed with PBS (-) were fixed in 4% PFA and 0.05%
504 glutaraldehyde in PBS (-) for 2.5 h. The immunogold labeling followed the method of Akagi *et al.*
505 ([Akagi et al., 2006](#)), except for the antibodies. After blocking with 10% normal goat serum, sections
506 were incubated in Tris-buffered saline (TBS) containing antibody against EV71 for 24–48 hours at
507 4°C and then washed with TBS and incubated with gold particle-conjugated secondary antibodies for
508 2 hours at room temperature. Thereafter, the sections were rinsed and embedded with a mixture of
509 1% polyvinyl alcohol containing 0.1% uranyl acetate, dried, and observed with an electron
510 microscope (Hitachi H-7650).

511 **Statistical analysis**

512 Statistical analysis was done using GraphPad Prism 7.00 (GraphPad Software Inc.). A
513 two-tailed unpaired *t*-test has been performed in Figs. 1D, 3B, S2A, 6B and 7B, and a two-tailed
514 Fisher's exact test has been performed in Figs. S1C and S2C.

515

516

Acknowledgements

517

518 We thank Dr. S. Koike (TMiMS) for the primary project assignment for S.O., allowing S.O.
519 to be a member of the laboratory, allowing S.O. to use facilities and the laboratory area, and
520 generously host S.O. instruments and samples. We thank Dr. K. Fujii (TMiMS) for generously
521 gifting RD-hSCARB2 cells, and Dr. H. Shimizu and Dr. M. Arita (National Institute of Infectious
522 Diseases, Japan) for kindly providing the virus strains and GFP-EV71 plasmid, respectively. We
523 appreciate Prof. K.T. Wong giving an opportunity for S.H.T. to engage in research with S.O. and Dr.
524 K. Ikeda (TMiMS) for generously allowing to prepare and publish this manuscript.

525

526

Competing interests

527

528

The authors declare no competing or financial interests.

529

530

Author contributions

531

532 S.O. was involved in project planning. S.O., S.H.T. and K.I. were involved in
533 experimental work and data analysis. E.I., K.O., K. Hagiwara and T.H. performed the experiments.
534 C.L. and P.C.C. provided the monoclonal antibody against EV71. K. Hanaki. and G.S. were
535 involved in data analysis. S.O. wrote the manuscript, which was revised by G.S., P.C.C., K. Hanaki
536 and S.O.

537

538

Funding

539

540 This work was supported in part by project research funding for Neurovirology Project
541 from TMiMS, a Grant-in-aid for Scientific Research (C) (25460583) from the Japan Society for the
542 Promotion of Science (S.O.), and in part by The Naito Foundation Subsidy for Female Researchers
543 after Maternity Leave (S.O.). S.H.T. was supported by High Impact Research
544 UM.C/625/1/HIR/MOHE/MED/06 (UM.0000064/HIR.C1) from the Ministry of Higher Education,
545 Malaysia government. G.S. is supported by Wellcome Senior Investigator Awards (107116/Z/15/Z
546 and 223022/Z/21/Z), and a UK Dementia Research Institute Foundation award (UKDRI-1005).

547

References

- 548
549
- 550 **Akagi, T., Ishida, K., Hanasaka, T., Hayashi, S., Watanabe, M., Hashikawa, T. & Tohyama, K.**
551 (2006). Improved methods for ultracryotomy of CNS tissue for ultrastructural and
552 immunogold analyses. *J Neurosci Methods*, **153**, 276-282.
- 553 **Arita, M., Koike, S., Aoki, J., Horie, H. & Nomoto, A.** (1998). Interaction of poliovirus with its
554 purified receptor and conformational alteration in the virion. *J Virol*, **72**, 3578-3586.
- 555 **Blanz, J., Groth, J., Zachos, C., Wehling, C., Saftig, P. & Schwake, M.** (2010). Disease-causing
556 mutations within the lysosomal integral membrane protein type 2 (LIMP-2) reveal the nature
557 of binding to its ligand beta-glucocerebrosidase. *Hum Mol Genet*, **19**, 563-572.
- 558 **Blomberg, J., Lycke, E., Ahlfors, K., Johnsson, T., Wolontis, S. & Von Zeipel, G.** (1974). Letter:
559 New enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and
560 mouth disease. *Lancet*, **2**, 112.
- 561 **Chen, P., Song, Z., Qi, Y., Feng, X., Xu, N., Sun, Y., Wu, X., Yao, X., Mao, Q., Li, X., Dong, W.,**
562 **Wan, X., Huang, N., Shen, X., Liang, Z. & Li, W.** (2012). Molecular determinants of
563 enterovirus 71 viral entry: cleft around GLN-172 on VP1 protein interacts with variable region
564 on scavenger receptor B 2. *J Biol Chem*, **287**, 6406-6420.
- 565 **Chin, T. M., Boopathy, G. T. K., Man, E. P. S., Clohessy, J. G., Csizmadia, E., Quinlan, M. P.,**
566 **Putti, T., Wan, S. C., Xie, C., Ali, A., Wai, F. C., Ong, Y. S., Goh, B. C., Settleman, J.,**
567 **Hong, W., Levantini, E. & Tenen, D. G.** (2020). Targeting microtubules sensitizes drug
568 resistant lung cancer cells to lysosomal pathway inhibitors. *Theranostics*, **10**, 2727-2743.
- 569 **Cuervo, A. M. & Dice, J. F.** (1996). A receptor for the selective uptake and degradation of proteins
570 by lysosomes. *Science*, **273**, 501-503.
- 571 **Dang, M., Wang, X., Wang, Q., Wang, Y., Lin, J., Sun, Y., Li, X., Zhang, L., Lou, Z., Wang, J.**

572 **& Rao, Z.** (2014). Molecular mechanism of SCARB2-mediated attachment and uncoating of
573 EV71. *Protein Cell*, **5**, 692-703.

574 Dikic, I. (ed.) 2006. *Sorting to the Biosynthetic Pathway*: Springer.

575 **Ebato, C., Uchida, T., Arakawa, M., Komatsu, M., Ueno, T., Komiya, K., Azuma, K., Hirose, T.,**
576 **Tanaka, K., Kominami, E., Kawamori, R., Fujitani, Y. & Watada, H.** (2008). Autophagy
577 is important in islet homeostasis and compensatory increase of beta cell mass in response to
578 high-fat diet. *Cell Metab*, **8**, 325-332.

579 **Eskelinen, E. L.** (2006). Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy. *Mol*
580 *Aspects Med*, **27**, 495-502.

581 **Eskelinen, E. L., Tanaka, Y. & Saftig, P.** (2003). At the acidic edge: emerging functions for
582 lysosomal membrane proteins. *Trends Cell Biol*, **13**, 137-145.

583 **Hagiwara, A., Tagaya, I. & Yoneyama, T.** (1978). Epidemic of hand, foot and mouth disease
584 associated with enterovirus 71 infection. *Intervirology*, **9**, 60-63.

585 **Hussain, K. M., Leong, K. L., Ng, M. M. & Chu, J. J.** (2011). The essential role of clathrin-
586 mediated endocytosis in the infectious entry of human enterovirus 71. *J Biol Chem*, **286**, 309-
587 321.

588 **Juenemann, K. & Reits, E. A.** (2012). Alternative macroautophagic pathways. *Int J Cell Biol*, **2012**,
589 189794.

590 **Kirkegaard, T.** (2013). Emerging therapies and therapeutic concepts for lysosomal storage diseases.
591 *Expert Opinion on Orphan Drugs*, **1**, 385-404.

592 **Lee, Y. R., Wang, P. S., Wang, J. R. & Liu, H. S.** (2014). Enterovirus 71-induced autophagy
593 increases viral replication and pathogenesis in a suckling mouse model. *J Biomed Sci*, **21**, 80.

594 **Lin, Y. W., Lin, H. Y., Tsou, Y. L., Chitra, E., Hsiao, K. N., Shao, H. Y., Liu, C. C., Sia, C., Chong,**
595 **P. & Chow, Y. H.** (2012). Human SCARB2-mediated entry and endocytosis of EV71. *PLoS*

596 *One*, **7**, e30507.

597 **Mcminn, P. C.** (2002). An overview of the evolution of enterovirus 71 and its clinical and public
598 health significance. *FEMS Microbiol Rev*, **26**, 91-107.

599 **Nagata, N., Shimizu, H., Ami, Y., Tano, Y., Harashima, A., Suzaki, Y., Sato, Y., Miyamura, T.,**
600 **Sata, T. & Iwasaki, T.** (2002). Pyramidal and extrapyramidal involvement in experimental
601 infection of cynomolgus monkeys with enterovirus 71. *J Med Virol*, **67**, 207-216.

602 **Nakajima, N., Kitamori, Y., Ohnaka, S., Mitoma, Y., Mizuta, K., Wakita, T., Shimizu, H. &**
603 **Arita, M.** (2012). Development of a transcription-reverse transcription concerted reaction
604 method for specific detection of human enterovirus 71 from clinical specimens. *J Clin*
605 *Microbiol*, **50**, 1764-1768.

606 **Nakata, K., Takeda, S., Tanaka, A., Kwang, J. & Komano, J.** (2017). Antiviral activity of acid
607 beta-glucosidase 1 on enterovirus 71, a causative agent of hand, foot and mouth disease. *J*
608 *Gen Virol*, **98**, 643-651.

609 **Nishida, Y., Arakawa, S., Fujitani, K., Yamaguchi, H., Mizuta, T., Kanaseki, T., Komatsu, M.,**
610 **Otsu, K., Tsujimoto, Y. & Shimizu, S.** (2009). Discovery of Atg5/Atg7-independent
611 alternative macroautophagy. *Nature*, **461**, 654-658.

612 **Nour, A. M. & Modis, Y.** (2014). Endosomal vesicles as vehicles for viral genomes. *Trends Cell Biol*,
613 **24**, 449-454.

614 **Pfeffer, S. R.** (2017). Rab GTPases: master regulators that establish the secretory and endocytic
615 pathways. *Mol Biol Cell*, **28**, 712-715.

616 **Plevka, P., Perera, R., Cardoso, J., Kuhn, R. J. & Rossmann, M. G.** (2012). Crystal structure of
617 human enterovirus 71. *Science*, **336**, 1274.

618 **Racaniello, V.** (2007). *Fields Virology, 5th ed.*, Philadelphia, PA, Lippincott Williams & Wilkins.

619 **Reczek, D., Schwake, M., Schroder, J., Hughes, H., Blanz, J., Jin, X., Brondyk, W., Van Patten,**

620 **S., Edmunds, T. & Saftig, P.** (2007). LIMP-2 is a receptor for lysosomal mannose-6-
621 phosphate-independent targeting of beta-glucocerebrosidase. *Cell*, **131**, 770-783.

622 **Rossmann, M. G. & Johnson, J. E.** (1989). Icosahedral RNA virus structure. *Annu Rev Biochem*,
623 **58**, 533-573.

624 **Semerdjieva, S., Shortt, B., Maxwell, E., Singh, S., Fonarev, P., Hansen, J., Schiavo, G., Grant,**
625 **B. D. & Smythe, E.** (2008). Coordinated regulation of AP2 uncoating from clathrin-coated
626 vesicles by rab5 and hRME-6. *J Cell Biol*, **183**, 499-511.

627 **Shimizu, H., Utama, A., Yoshii, K., Yoshida, H., Yoneyama, T., Sinniah, M., Yusof, M. A., Okuno,**
628 **Y., Okabe, N., Shih, S. R., Chen, H. Y., Wang, G. R., Kao, C. L., Chang, K. S., Miyamura,**
629 **T. & Hagiwara, A.** (1999). Enterovirus 71 from fatal and nonfatal cases of hand, foot and
630 mouth disease epidemics in Malaysia, Japan and Taiwan in 1997-1998. *Jpn J Infect Dis*, **52**,
631 12-15.

632 **Song, J., Hu, Y., Li, J., Zheng, H., Wang, J., Guo, L., Shi, H. & Liu, L.** (2018). Suppression of the
633 toll-like receptor 7-dependent type I interferon production pathway by autophagy resulting
634 from enterovirus 71 and coxsackievirus A16 infections facilitates their replication. *Arch Virol*,
635 **163**, 135-144.

636 **Stenmark, H.** (2009). Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol*, **10**,
637 513-525.

638 **Sun, N., Yun, J., Liu, J., Malide, D., Liu, C., Rovira, Ii, Holmstrom, K. M., Fergusson, M. M.,**
639 **Yoo, Y. H., Combs, C. A. & Finkel, T.** (2015). Measuring In Vivo Mitophagy. *Mol Cell*, **60**,
640 685-696.

641 **Thompson, S. R. & Sarnow, P.** (2003). Enterovirus 71 contains a type I IRES element that functions
642 when eukaryotic initiation factor eIF4G is cleaved. *Virology*, **315**, 259-266.

643 **Vega, M. A., Segui-Real, B., Garcia, J. A., Cales, C., Rodriguez, F., Vanderkerckhove, J. &**

644 **Sandoval, I. V.** (1991). Cloning, sequencing, and expression of a cDNA encoding rat LIMP
645 II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36.
646 *J Biol Chem*, **266**, 16818-16824.

647 **Wandinger-Ness, A. & Zerial, M.** (2014). Rab proteins and the compartmentalization of the
648 endosomal system. *Cold Spring Harb Perspect Biol*, **6**, a022616.

649 **Wang, X., Peng, W., Ren, J., Hu, Z., Xu, J., Lou, Z., Li, X., Yin, W., Shen, X., Porta, C., Walter,**
650 **T. S., Evans, G., Axford, D., Owen, R., Rowlands, D. J., Wang, J., Stuart, D. I., Fry, E. E.**
651 **& Rao, Z.** (2012). A sensor-adaptor mechanism for enterovirus uncoating from structures of
652 EV71. *Nat Struct Mol Biol*, **19**, 424-429.

653 **Yamayoshi, S. & Koike, S.** (2011). Identification of a human SCARB2 region that is important for
654 enterovirus 71 binding and infection. *J Virol*, **85**, 4937-4946.

655 **Yamayoshi, S., Ohka, S., Fujii, K. & Koike, S.** (2013). Functional comparison of SCARB2 and
656 PSGL1 as receptors for enterovirus 71. *J Virol*, **87**, 3335-3347.

657 **Yamayoshi, S., Yamashita, Y., Li, J., Hanagata, N., Minowa, T., Takemura, T. & Koike, S.** (2009).
658 Scavenger receptor B2 is a cellular receptor for enterovirus 71. *Nat Med*, **15**, 798-801.

659 **Zachos, C., Blanz, J., Saftig, P. & Schwake, M.** (2012). A critical histidine residue within LIMP-2
660 mediates pH sensitive binding to its ligand beta-glucocerebrosidase. *Traffic*, **13**, 1113-1123.

661 **Zhao, Y., Ren, J., Padilla-Parra, S., Fry, E. E. & Stuart, D. I.** (2014). Lysosome sorting of beta-
662 glucocerebrosidase by LIMP-2 is targeted by the mannose 6-phosphate receptor. *Nat Commun*,
663 **5**, 4321.

664

665

666

Figure legends

667

668 **Figure 1. EV71 is uncoated at 30 m.a.i. and is dependent on acidification**

669 (A) Experimental protocol for infection of RD-hSCARB2 cells with light-sensitive EV71. The red
670 arrow indicates virus addition, the green arrow indicates the recovery of the infected cells followed
671 by the titration of the live virus, black arrowheads indicate the start of light irradiation, gray areas
672 indicate the periods without the light irradiation, and yellow area indicates the periods with the light
673 irradiation. The time after the infection with light-sensitive virus is indicated. (B) Virus titer of
674 the recovered virus. Horizontal axis indicates the onset of light irradiation after infection. The top
675 line represents the virus titer without irradiation. Error bars represent SD. (C) In situ hybridization
676 for RNA genome of EV71 and immunofluorescence for the capsid proteins after infection of RD-
677 hSCARB2 cells with EV71 in the absence or presence of NH₄Cl. The time of fixation after infection
678 is indicated. DAPI is in blue, EV71 RNA genome is in green, whilst EV71 capsid antigens are in
679 red. The panels on the right are enlarged 3D cross-section views of the dashed rectangles in the
680 merged panels. Arrowheads indicate EV71 RNA + capsid double-labelled clustered vesicles.
681 Insets are 400% enlarged panels of the arrowhead areas. Representative images are shown. Scale
682 bar, 10 µm. (D) The numbers of visible green dots corresponding to EV71 RNA genomes per cell
683 were shown. Number of z stack sets was three except for the 30 min NH₄Cl+ sample (two). **, *P*
684 < 0.01. Statistical significance was determined by a two-tailed, unpaired *t*-test. Error bars
685 represent s.d.

686

687 **Figure 2. EV71 co-distributes with hSCARB2 and M6PR, but not with LAMP1 until 40 m.a.i.**

688 Co-localization of EV71 and hSCARB2 (A), EEA1 (B), M6PR (C), or LAMP1 is shown by
689 immunofluorescence (D). RD-hSCARB2 cells were infected with EV71 and fixed 30 minutes (B-

690 D) or 40 minutes (A) after infection followed by immunofluorescence. DAPI is in blue, EV71 antigen
691 is in green, whereas hSCARB2 or other markers are in red. The panels on the right are enlarged 3D
692 cross-section views of the dashed rectangles in the merged panels. Arrowheads indicate co-
693 localization of EV71 with markers. Representative images are shown. Scale bars, 10 μm . (E)
694 Kinetics of co-localization of EV71 with hSCARB2 or other markers. Times indicate the time after
695 infection.

696

697 **Figure 3. EGF co-localizes with LAMP1 at 30 m.a.i.**

698 (A) Co-localization of EGF with M6PR, LAMP1 or SCARB2 is shown by immunofluorescence of
699 EGF and M6PR, LAMP1 and SCARB2. RD-hSCARB2 cells were infected with EV71 and then
700 fixed 30 minutes after the addition of EGF followed by immunofluorescence DAPI is in blue, EGF
701 is in green, whereas organelle markers are in red. The panels on the right are enlarged 3D cross-
702 section views of the dashed rectangles in the merged panels. Arrowheads indicate co-localization
703 of EGF with markers. Representative images are shown. Scale bar, 10 μm . (B) Quantification
704 of co-localization of EGF with specific markers. Number of z stack sets was two except SCARB2
705 was three. ns, not significant, **, $P < 0.01$. Statistical significance was determined by a two-tailed,
706 unpaired *t*-test. Error bars represent s.d.

707

708 **Figure 4. EV71 co-distributes with RAB9 during the uncoating period**

709 Co-localization of EV71 with RAB5, RAB7, or RAB9 is shown by immunofluorescence of EV71
710 and RAB5 (A), RAB7 (B), or RAB9 (C). RD-hSCARB2 cells were infected with EV71 and fixed
711 30 minutes after infection, followed by immunofluorescence. DAPI is in blue, EV71 antigen is in
712 green, whereas marker antigens are in red. The panels on the right are enlarged 3D cross-section
713 views of the dashed rectangles in the merged panels. Arrowheads indicate co-localization of EV71

714 with markers. Representative images are shown. Scale bar, 10 μ m. (D) Kinetics of co-
715 localization of EV71 with the different markers. Times indicate the time after infection.

716

717 **Figure 5. EV71 particles are incorporated into LEs**

718 (A) Transmission electron microscopy. RD-hSCARB2 cells were infected with EV71 and fixed at
719 20 or 30 m.a.i. Times indicate the time after infection. Arrowheads indicate representative EV71-
720 like particles in LE. (B) Immuno-electron microscopy. Gold particles label EV71 antigen.
721 Arrowheads indicate representative gold particles existed in the lumen of single membrane
722 compartments without ILV at 20 m.a.i., and representative gold particles accumulated in single
723 membrane organelles containing ILVs at 30 m.a.i. The experiments were repeated two times and
724 the same trends were observed. Scale bar, 200 nm.

725

726 **Figure 6. EV71 translocates into BMP-enriched LE at 30 m.a.i.**

727 (A) RD-hSCARB2 cells were infected with EV71 and then fixed 20 minutes or 30 minutes after the
728 infection followed by immunofluorescence. DAPI is in blue, EV71 antigen is in green, whereas
729 BMP is labelled in red. The panels on the right are enlarged 3D cross-section views of the dashed
730 rectangles in the merged panels. Arrowheads indicate co-localization of EV71 with BMP.
731 Representative images are shown. Scale bar, 10 μ m. (B) Co-localization rate of EV71 with BMP
732 at 20 or 30 m.a.i. **, $P < 0.01$. Statistical significance was determined by a two-tailed, unpaired
733 *t*-test. Error bars represent s.d.

734

735 **Figure 7. CD-M6PR is essential for EV71 replication**

736 (A) Western blotting of RAB5, RAB7, RAB9, CD-M6PR, CI-M6PR and clathrin heavy chain in cells
737 in which these genes have been targeted by siRNA knockdown. The expression of β -actin was

738 examined as loading control. Triangles indicate molecular weight markers. Black dots indicate
739 the predicted molecular weight of the proteins. The numbers are the densities and the ratio of anti-
740 markers/anti β -actin. (B) RD cells were treated with siRNAs targeting RAB5, RAB7, RAB9, CD-
741 M6PR, CI-M6PR or clathrin heavy chain and then infected with EV71-GFP. Cells were fixed at 31
742 hours after infection, followed by fluorescent imaging. GFP-positive cells were counted and their
743 percentage against the total number of DAPI-positive cells plotted. *, $P < 0.05$. Statistical
744 significance was determined by a two-tailed, unpaired t -test. Error bars represent s.d.
745