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1 **Extracellular albumin and endosomal ions prime enterovirus particles**  
2 **for uncoating that can be prevented by fatty acid saturation**

3

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## 17 **Abstract**

18 There is limited information about the molecular triggers leading to the uncoating of enteroviruses  
19 in physiological conditions. Using real-time spectroscopy and sucrose gradients with radioactively-  
20 labeled virus we show at 37 °C, formation of a low amount of albumin-triggered, metastable,  
21 uncoating intermediate of echovirus 1 without receptor engagement. This conversion was blocked  
22 by saturating the albumin with fatty acids. High potassium but low sodium and calcium  
23 concentrations, mimicking the endosomal environment, also induced the formation of a metastable  
24 uncoating intermediate of echovirus 1. Together, these factors boosted the formation of the  
25 uncoating intermediate and infectivity of this intermediate was retained, as judged by end-point  
26 titration. Cryo-electron microscopy reconstruction of the virions treated with albumin and high  
27 potassium, low sodium and low calcium concentrations resulted in a 3.6 Å resolution model  
28 revealing a fenestrated capsid showing 4 % expansion and loss of the pocket factor, similarly to  
29 altered (A-) particles described for other enteroviruses. The dimer interface between VP2 molecules  
30 was opened, the VP1 N-termini disordered and most likely externalised. The RNA was clearly  
31 visible, anchored to the capsid. The results presented here suggest that extracellular albumin,  
32 partially saturated with fatty acids, likely leads to the formation of the infectious uncoating  
33 intermediate prior to the engagement with the cellular receptor. In addition, changes in mono- and  
34 divalent cations, likely occurring in endosomes, promote capsid opening and genome release.

## 35 **Importance**

36 There is limited information about uncoating of enteroviruses in physiological conditions. Here, we  
37 focused on physiologically relevant factors that likely contribute to opening of echovirus 1 and  
38 other B-group enteroviruses. By combining biochemical and structural data, we show, that before  
39 entering cells, extracellular albumin is capable of priming the virus into a metastable, yet infectious  
40 intermediate state. The ionic changes that are suggested to occur in endosomes, can further  
41 contribute to uncoating and promote genome release, once the viral particle is endocytosed.

42 Importantly, we provide a detailed high-resolution structure of a virion after treatment with albumin  
43 and a preset ion composition, showing pocket factor release, capsid expansion and fenestration, and  
44 the clearly visible genome still anchored to the capsid. This study provides valuable information  
45 about the physiological factors that contribute to the opening of B-group enteroviruses.

46

## 47 **Introduction**

48 The Enterovirus B species consists of tens of clinically relevant viruses, including over 30 serotypes  
49 of echoviruses, coxsackievirus B1-B6 and A9. They can cause a wide variety of mild and severe  
50 infections and many of them are also associated with the onset of type I diabetes and coeliac disease  
51 (1-4). These viruses infect primarily gut epithelial cells and are naturally stable in an acidic  
52 environment. This latter important feature makes the EV-B group viruses different from, for  
53 example, rhinoviruses that are known to rely on acidification during virus uncoating (5, 6).

54 Earlier we have shown that echovirus 1 (E1) and coxsackievirus A9 trigger a clathrin-independent  
55 entry pathway (7-10). Within 15 minutes of entry, viruses are first localized in the endosomes that  
56 develop into pH neutral multivesicular bodies. The genome uncoating continues for up to 2 hours  
57 post infection (8, 11, 12). A large number of host cell factors have been pinpointed as important  
58 regulators for the entry of enteroviruses (13). However, there is very little information on the  
59 possible extracellular and co-internalised soluble factors that potentially contribute to uncoating  
60 during infection.

61 Recently we described a novel uncoating intermediate particle of E1 which formed during infection  
62 and could be isolated from the cells at early stages of infection (14). This particle proved to be  
63 stable, infectious, containing all of its capsid proteins, and still capable of receptor binding (14).  
64 Previously, several studies have characterized uncoating intermediates for entero- and rhinoviruses  
65 that have been termed as A- or 135S-particles based on their altered conformation and lighter  
66 sedimentation in sucrose gradients (15). The formation of these particles has been suggested to be

67 induced by receptor binding (16, 17), low pH (18), or by non-physiological high temperatures (19).  
68 They have also been found to exist in purified virus preparations (20). One study showed that  
69 treatment with fatty acid free BSA converts echovirus 12 into A-particles, but the mechanism of  
70 action was not investigated further (21). In addition, there are only few studies reporting what other  
71 physiological factors, such as changes in ionic conditions, may cause on the virus particle (21-25).  
72 X-ray crystallography as well as cryo-electron microscopy (cryo-EM) and single particle  
73 reconstruction have been used to gain structural information on picornavirus particles at different  
74 stages before genome release (20, 26-33). Due to methodological challenges it has been difficult to  
75 exactly map the spatiotemporal events during the uncoating process and to link that to structural  
76 information. Furthermore, the physiological conditions in the tissues where virus infection takes  
77 place have not been carefully studied.

78 Here we show that albumin triggers the uncoating process of E1 at 37 °C, in a manner dependent  
79 upon the balance between fatty acids and albumin present. We further show that changes in mono-  
80 and divalent cations, likely reflecting the endosomal concentrations, also trigger a slower uncoating  
81 process of E1, which is clearly boosted by albumin.

## 82 **Results**

### 83 **Serum at physiological temperature drives transformation from intact E1 virion to an** 84 **uncoating intermediate**

85 In this study, we investigated physiologically relevant factors that promote the uncoating process of  
86 E1. First, we noticed that in PBS buffer E1 remained infectious up to 21 days at room temperature  
87 and, remarkably, at 37 °C, still some virus stayed infectious in such conditions after 5 days (Fig.  
88 1A). A three-hour incubation of E1 in PBS-MgCl<sub>2</sub> at 37 °C resulted in only a minor formation of  
89 empty virus particles and no formation of the uncoating intermediate, as detected by real-time  
90 fluorescent measurement in the presence or absence of RNase (Fig. 1B). This finding was further

91 confirmed by gradient centrifugation of  $^{35}\text{S}$  labelled E1 (Fig. 1C). The virus was even more stable  
92 in DPBS buffer (containing also  $\text{CaCl}_2$ , for buffer compositions see Table 1) throughout the 3-hour  
93 treatment (Fig. 1B). If enteroviruses are so stable, what molecules efficiently trigger uncoating in  
94 the right location, i.e. endosomes? We observed that conversion of E1 virions to intermediate  
95 particles was significantly enhanced by treating the virus with cell culture medium containing 1%  
96 serum (1% S-MEM): the SYBR Green II fluorescence increased considerably after 10 to 15  
97 minutes incubation at  $+37^\circ\text{C}$ , reaching maximal fluorescence around 40-50 minutes (Fig. 1D). At  
98 45 minutes, approximately 68% of the intensity originated from intermediate particles and 32%  
99 from empty capsids (Fig. 1D). The increase of both forms was again confirmed by sucrose gradient  
100 centrifugation using  $^{35}\text{S}$  E1 (Fig. 1E). A similar effect was also observed by fluorescence  
101 measurements of Coxsackievirus B3 and Coxsackievirus A9 (Fig. 1F) suggesting that this  
102 phenomenon was not restricted to E1. Virus conversion to the intermediate form was strictly  
103 temperature dependent, as 1% S-MEM did not induce changes in the virus capsid at room  
104 temperature but did induce the formation quickly after raising the temperature to  $37^\circ\text{C}$  (Fig. 1G).  
105 Altogether, these results show that, while E1 is stable in physiological buffers, 1% bovine serum  
106 effectively induces formation of the uncoating intermediate and empty capsids at  $37^\circ\text{C}$ .

107

### 108 **Ion composition mimicking endosomal conditions also triggers the E1 uncoating process**

109 Concerning ionic factors, uncoating of E1 and Coxsackievirus A9 is known to be independent of  
110 endosome acidification (13), which suggests that ion concentrations other than  $\text{H}^+$ , such as  $\text{K}^+$ ,  $\text{Na}^+$ ,  
111  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  might be more important for E1 uncoating inside the endosomes. We thus chose a  
112 combination of ion concentrations based on the measurements made from endosomes (34-38) and the  
113 information provided by Scott and Gruenberg in their review on endosomal ionic conditions (39)  
114 and tested their effect on promoting E1 uncoating. The "endosomal ionic solution" used in this  
115 study contained 20 mM  $\text{NaCl}$ , 30 mM  $\text{K}^+$ , 0.5 mM  $\text{MgCl}_2$  and 0.2 mM  $\text{CaCl}_2$  and is referred to as

116 NKMC. The spectroscopy results showed that the hypotonic NKMC solution promoted a slow  
117 formation of the uncoating intermediate within a 3 h period at 37 °C, as detected by the gradual  
118 increase of fluorescence to a roughly 4-times higher level when normalized to that of the DPBS  
119 treatment (Fig. 2A). Therefore, NKMC facilitates formation of mainly porous particles most of  
120 which still contain RNA. Increasing the concentration of  $K^+$  (60 mM) in relation to  $Na^+$  (20 mM)  
121 seemed to promote RNA release suggesting that increase of  $K^+$  found in the endosomes may be an  
122 important factor to promote the final RNA release (Fig. 2A). We also found higher amount of RNA  
123 release when we tested omitted the divalent cations in the buffer (i.e. NK solution containing 20  
124 mM NaCl and 30 mM  $K^+$  without  $Mg^{2+}$  or  $Ca^{2+}$ ). Without  $Mg^{2+}$  or  $Ca^{2+}$ , the fluorescence signal was  
125 mainly comprised of released RNA indicating the presence of mainly empty capsids (Fig. 2A). This  
126 is in line with the previously observed stabilizing effect of divalent cations on viral particles (24, 40-  
127 45) . The stabilizing effect of divalent cations was further verified by complementing the NK  
128 solution with different concentrations of  $Ca^{2+}$  or  $Mg^{2+}$  ions. Similarly to NK solution without  
129 divalent cations, concentrations of 0.002 and 0.02 mM  $Ca^{2+}$ , 100-fold and 10-fold dilutions of  $Ca^{2+}$   
130 compared to NKMC respectively, as well as 0.005 and 0.05 mM  $Mg^{2+}$ , 100-fold and 10-fold  
131 dilutions of  $Mg^{2+}$  compared to NKMC respectively, mainly caused formation of empty virions (Fig  
132 2B and C). In contrast, the presence 0.2 mM  $Ca^{2+}$  or 0.5 mM  $Mg^{2+}$  in NK solution prevented RNA  
133 release from a significant number of the virions (Fig 2B and C, black lines).

134 We also tested different concentrations of sodium and potassium ranging between their extra- and  
135 intracellular values (extracellular concentrations roughly 140 mM  $Na^+$  and 5 mM  $K^+$  versus  
136 intracellular 5 mM  $Na^+$  and 140 mM  $K^+$ ). The concentration of  $Mg^{2+}$  and  $Ca^{2+}$  were kept at their  
137 cytoplasmic values of 0.8 mM and 0.2  $\mu$ M, respectively, while we changed  $Na^+$  and  $K^+$   
138 concentrations step-by-step from 5 mM to 140 mM and 140 mM to 5 mM, respectively. The results  
139 showed that, at 37 °C, cytoplasmic ionic concentrations caused a clear increase in the number of  
140 empty virus particles: RNA release started already within 15 min (Fig. 2D). This suggests that the

141 intracellular ion concentrations, 140 mM  $K^+$ , 5mM  $Na^+$ , 0.5 mM  $MgCl_2$  and 0.2  $\mu M$   $Ca^{2+}$  are  
142 effective in promoting RNA release. Notably, the extracellular concentrations of  $Na^+$  (high) and  $K^+$   
143 (low) resulted in formation of empty particles in the presence of intracellular  $Mg^{2+}$  (0.5mM) and  
144  $Ca^{2+}$  (0.2 $\mu M$ ) concentrations (Fig. 2D). The concentrations of  $K^+$  and  $Na^+$  between the extremes  
145 were less effective at promoting RNA release (Fig. 2E-F).

146 In summary, the ionic conditions found in endosomes, with lowered  $Na^+$  and  $Ca^{2+}$  and higher  $K^+$   
147 concentrations as compared to the extracellular space, trigger a slow uncoating process. Even  
148 further reduction in the  $Ca^{2+}$  concentration and increase in  $K^+$  concentration, as may happen in the  
149 endosomes, facilitates also the RNA release from the virions.

150

#### 151 **Albumin triggers the E1 uncoating process**

152 Comparison of 1% S-MEM and MEM without serum clearly showed that serum triggered the  
153 uncoating process resulting in an increase of both the uncoating intermediate and empty virus (Fig.  
154 3A). We then tested if serum would have any additive effect when administrated in the NKMC  
155 solution. We observed a clear increase in the rate of the fluorescence signal appearance: with  
156 NKMC, the maximal fluorescence signal was reached only at the end of the 3 h measurement,  
157 whereas in the presence of 1% serum this time was shortened to about 30 min (Fig. 3B).  
158 Interestingly, with 1% serum, the amount of RNA released from the virions decreased and the  
159 intermediate particles remained stable throughout the measurement.

160 In order to narrow down the serum components responsible for the boosting effect, we focused on  
161 albumin, as it is the most abundant protein in serum (46). As 1% serum corresponds to  
162 approximately 0.04% albumin solution, we decided to use 0.01-0.1% BSA concentrations to see if  
163 albumin was responsible for the changes that we observed with 1% S-MEM. As it is known that  
164 albumin is a high affinity fatty acid carrier in the blood (46), and the lipid moiety, typically palmitate

165 (47), present in the hydrophobic pocket of many enteroviruses is important for the virion stability  
166 (15), we tested also fatty acid free BSA (faf-BSA) in our experiments.

167 The spectroscopy measurements showed that both BSA and faf-BSA triggered the uncoating  
168 process in MEM in a similar manner to 1% S-MEM (Fig. 3A and 3C), suggesting that the major  
169 factor in serum initiating the uncoating process is indeed the serum albumin. Moreover, both  
170 albumin forms promoted serum-like effects in NKMC too (Fig. 3D). After observing, that BSA and  
171 faf-BSA resulted in similar results as serum in both isotonic MEM and hypotonic NKMC, we tested  
172 faf-BSA in DPBS and saw that it induced the uncoating process similarly to MEM (Fig. 3E).  
173 Spectroscopy analysis of the virus treated with EM buffer prepared for cryoEM imaging, showed a  
174 high amount of the uncoating intermediate particles (Fig. 3E).

175 Since albumin appeared to be the major factor inducing and boosting the virus priming, we tested if  
176 addition of fatty acids would prevent the observed effect. We started with conditions that most  
177 efficiently caused RNA release: We treated the <sup>35</sup>S-labelled E1 with NK solution supplemented  
178 with 0.1% faf-BSA (Fig. 3F, red curve peak fractions 4-9). The presence of approximately 100-fold  
179 molar excess of palmitate (400  $\mu$ M) with respect to the amount of albumin in the assay, fully  
180 protected the E1 virions from the structural changes as detected by sucrose gradient analysis (Fig.  
181 3F, blue curve).

182 We next explored in more detail the molar ratio between BSA and virus required for efficient  
183 triggering of the uncoating process. We found that albumin to virion ratio of 1200:1 (comparable to  
184 0,01% faf-BSA solution with 1 $\mu$ g of E1 used in spectroscopy assays), which corresponds to  
185 albumin ratio to hydrophobic pocket of 20:1, efficiently triggered the formation of the uncoating  
186 intermediate (Fig. 3G). In contrast, albumin to virion ratio of 120:1 showed only a mild effect and  
187 ratio 17:1 induced no changes when compared to the control condition (data not shown). The virus  
188 treatment with albumin did not significantly increase the amount of empty viruses.

189 Then we tested the concentration of free fatty acids needed to prevent the uncoating process.  
190 Adding increasing amounts of palmitate to the faf-BSA virus mixture reduced the formation of  
191 uncoating intermediate and concomitantly increased the amount of intact virions (Fig. 3G). Full  
192 protection against uncoating was gained by adding a 50-fold molar excess of palmitate with respect  
193 to the faf-BSA molecules, whereas a 20-fold excess showed an intermediate effect (Fig. 3G). The  
194 effect of a 10-fold excess was still notable but an equimolar ratio showed no protection (data not  
195 shown).

196 These results show that albumin is the major component of the serum that triggers the uncoating  
197 process. It further stresses the fact that the net balance between fatty acids and albumin is important  
198 in enterovirus particle uncoating.

199

#### 200 **Structural details of the treated virion**

201 Having ascertained the physiological factors that could reproducibly start the uncoating process, we  
202 studied the treated virions by negative staining as well as by cryoEM and single particle  
203 reconstruction. Negatively-stained preparations of cryoEM buffer treated sample revealed three  
204 distinct forms of E1: intact, intermediate and empty particles (Fig. 4A). In the EM buffer treated  
205 virus sample, 29% of particles were intact, 65% were intermediate and 6% were empty (out of  
206 2108), whereas in non-treated sample the percentages were 95%, 4% and 1% (out of 2624),  
207 respectively. In cryoEM micrographs we could not distinguish between intact and the intermediate  
208 particles by eye, but after image analysis and classification 70 % of all particles were intermediate  
209 (23983 out of 34160) processed from the treated sample's micrographs (Fig. 4B). Thus, the  
210 reconstruction of the treated particle described below represents the averaged structure for the most  
211 populated viral particle in the treated sample. Two-dimensional averages of the control and treated  
212 particles clearly show the density for both the capsid and RNA (Fig 4C).

213 Icosahedral reconstruction of the intact E1 virus from the control sample's cryoEM micrographs to  
214 3.5 Å resolution, was similar to the published X-ray structure PDB ID 1EV1 (48) . The atomic

215 model fitted into the intact E1 reconstruction included the lipid factor in the lipid pocket and density  
216 for all four capsid proteins, VP1, VP2, VP3 and VP4 (Table 3, Fig. 4D, F, G, H and Fig. 5A-C) (48).  
217 The RMSD compared to the X-ray structure was 0.77 Å. The intact E1 reconstruction shows  
218 icosahedrally-averaged RNA density which has not been reported earlier (Fig. 4F and H, Fig. 5A).  
219 The majority of the RNA follows the outline of the capsid and is distributed at a distance of 0 – 10  
220 Å from the inner capsid surface, with the highest density between radii 94 and 113 Å (from the  
221 virion center). The RNA has clear connections around the two-fold axes of symmetry to Trp 38 of  
222 VP2 (Fig. 5A). In addition, Arg 13 and Arg 27 of VP1 as well as a poorly resolved VP4 loop  
223 contact the RNA density. Similar RNA-capsid interactions involving VP2 Trp 38 and VP1 N-  
224 terminus have been described in intact rhinovirus particles (30).

225 In comparison, the 3.6 Å resolution treated particle reconstruction has undergone a 4% expansion  
226 (Fig. 4E, I, J, K). It was possible to model most of the VP1, VP2 and VP3, but none of VP4 in to  
227 the reconstruction (Table 3 and Fig. 5D-F). The atomic modelling revealed that the capsid  
228 expansion occurred through rotation and outwards translation of the capsid proteins VP1, VP2 and  
229 VP3. This results in a fenestrated capsid, with prominent holes on the edges of the capsid close to  
230 the two-fold axes of symmetry at the VP2 dimer interface, a hallmark of A-particles described for  
231 other enteroviruses (Fig. 4J and Fig. 6A-B). The atomic model emphasizes these holes, somewhat  
232 artificially, as not all the electron density has been accounted for (Fig. 6B). The pocket factor has  
233 been released (Fig. 5E and F). The GH loop on VP1, thought to be important in pocket factor  
234 release has moved, collapsing the pocket. The RNA is clearly visible, its average conformation has  
235 changed, but it still maintains connections to the capsid via VP2 Trp 38, Glu 40 and Tyr 41 (Fig.  
236 5D). The contacts mediated by the VP1 N-termini and VP4 have been lost in the treated particle,  
237 but new connections appeared via the N-termini of VP3 below the five-fold vertices. In the control  
238 particle these VP3 termini interact with VP4. Similar RNA-capsid interactions mediated by VP2  
239 Trp 38 and VP3 N-termini were seen in expanded rhinovirus particles (30). The averaged RNA still

240 follows the profile of the inner surface of the capsid, but has moved outwards (Fig. 4, 5 and 6), with  
241 the highest density between radii 102 and 122Å. Thus, the RNA is less densely packed in the  
242 treated intermediate particle.

243 Besides the poorly-ordered density inside the capsid assigned to viral RNA, the difference map  
244 between the control reconstruction and the atomic model of intact E1 showed only a small  
245 unassigned density in the capsid protein region at the three-fold axes of symmetry (Fig. 6C). This  
246 unassigned density could be attributed to six un-modelled residues of the VP2 N-terminus (Fig. 6C,  
247 red arrows in the inset). The difference map between the treated particle reconstruction and atomic  
248 model, revealed, less-well ordered, lower resolution density near the two-fold axes spanning the  
249 capsid from the interior to the exterior, and at the five-fold axes on the particle surface (Fig. 6D).

250 The first modelled residue of the VP1 N-terminus (Asn 55) lies in close proximity to the unassigned  
251 density near the two-fold axis inside the particle and therefore, the unassigned density could be the  
252 VP1 N-termini now traversing the capsid and exposed on the surface of the treated capsid similarly  
253 to the interpretation for expanded particles of other enteroviruses (Fig. 6D, red arrows in the inset)  
254 (20, 26, 49). In addition, part of this poorly-ordered density seen on the exterior could be attributed to  
255 a flexible VP3 loop (residues Thr 175 – Asp 183). The poorly defined density seen at the five-fold  
256 axes on the capsid surface is most likely attributed to disordered loops in VP1 (residues Thr 131 –  
257 Asn 136). Although VP4 could not be modelled in the density, its partial presence in the treated  
258 sample was confirmed by autoradiography (Fig. 7). Moreover, the treated virus sample showed  
259 about 2 logs lower infectivity (decrease from  $8.23 \times 10^{11}$  to  $1.01 \times 10^{10}$ ) confirming our previously  
260 published data for the E1 uncoating intermediate particle (14). Hence, VP4 could contribute to both  
261 the poorly-ordered density on the inside of the capsid close to the vertices, attributed primarily to  
262 RNA, as well as to the density spanning the capsid. In corroboration of this finding, the presence of  
263 a tiny amount of VP4 was recently reported in A-particles of enterovirus D68 induced by acid  
264 treatment (49).

265 **Discussion**

266 We have shown previously that during entry into cells, E1 undergoes structural changes that were  
267 first discovered as an increased permeability to the small molecule dye, SYBR Green II, and Cs-  
268 ions (14). Here, we showed by cryoEM that temperature-dependent structural changes, under  
269 physiological conditions, involved expansion of the virus particle, loosening of the genome  
270 packing, loss of the lipid factor and formation of larger openings at the VP2 dimer interface,  
271 explaining the increased permeability to the small molecular dye and the resistance to RNase  
272 treatment observed previously (14). We also showed by spectroscopy that serum-priming of  
273 coxsackievirus B3 and coxsackievirus A9 caused similar changes in permeability to that of E1  
274 suggesting that also other enteroviruses behave similarly (Fig. 1F).

275 Our *in vitro* experiments demonstrated two important factors in the serum and extracellular space  
276 affecting the integrity and the dynamics of E1 virion at 37 °C, albumin and fatty acids, one of the  
277 most abundant components in the serum and interstitial fluids. Albumin concentration in serum has  
278 been reported as 640  $\mu\text{M}$  (46) and the albumin bound fatty acids concentration ranging from 200 to  
279 700  $\mu\text{M}$  in serum (50). As mentioned before, albumin serves as the major fatty acid carrier in the  
280 blood with two to three high affinity and four to five intermediate affinity binding sites for fatty  
281 acids (46). Also, the fatty acid binding capacity of BSA and faf-BSA have been measured to be  
282 around 4.8 moles and 7 moles per mole albumin, respectively (51). The measurements further  
283 showed that BSA is approximately 50% occupied by serum fatty acids, meaning that roughly 50%  
284 of the fatty acid binding capacity is still left (51). Furthermore, both Penn et al. (51) and Van der  
285 Vusse (46) showed that bovine and human albumin have very similar fatty acid binding capacities  
286 making bovine albumin a good surrogate for human albumin. These data provide further support,  
287 that in physiological conditions, BSA is not saturated with fatty acids. Thus, the most likely  
288 explanation for the increased intermediate particle formation at low fatty acid concentration is the  
289 partitioning of the fatty acid moiety from the capsid into a more hydrophobic environment, such as

290 into the fatty acid binding sites on the albumin. Higher temperature will result in higher mobility of  
291 the fatty acid. Furthermore, the fatty acid saturation level of albumin has a great effect on capsid  
292 stability. This was demonstrated by the prevention of virus expansion and RNA release using  
293 increasing concentrations of palmitate in comparison to albumin (Figs. 3E and 3F). Considering the  
294 observed molar excess of fatty acids required to prevent formation of albumin-induced intermediate  
295 particle (Fig. 3F), and the approximately 1:1 ratio of albumin and fatty acids observed in serum, it  
296 seems probable that in physiological conditions, the albumin present outside the cells starts the  
297 uncoating process. The ability of faf-BSA to induce echovirus A-particle formation was previously  
298 shown using a radioactive gradient, however the nature of this process was not studied further (21).  
299 Our results here explain the mechanism by which faf-BSA induces uncoating in enteroviruses, such  
300 as echoviruses and coxsackieviruses. Moreover, we suggest that the loss of the pocket factor likely  
301 precedes receptor binding, promoting the formation of an infectious intermediate particle already in  
302 the extracellular space.

303 The expansion of E1 capsid does not affect the receptor,  $\alpha_2$ I-domain, binding site (14, 52). However,  
304 the expansion does cause changes in the amino acid composition exposed on the capsid surface  
305 such as the exposure of the VP1 N-terminus, which may also give rise to new secondary receptor-  
306 binding sites. If some lipid factors would still be bound to the capsid in the endosome initially, it  
307 could well exchange into the hydrophobic environment of the endosomal membrane that would  
308 extract the lipid factor from the capsid. In support of this hypothesis, albumin readily donates fatty  
309 acids to other fatty acid binding proteins in the vicinity of the plasma membrane. Furthermore, the  
310 binding and release of the lipid factor in enteroviruses is known to be a dynamic process (53, 54). A  
311 potential endosomal membrane protein that could help to sequester the lipid factor is the lipid-  
312 modifying enzyme PLA2G16 (55). It is thought to affect the insertion of VP4 into the endosomal  
313 membrane, but could potentially help to sequester the lipid factor too (56).

314 The next and final step in picornavirus uncoating is the RNA release. Accumulated results on  
315 CVA9 and E1 uncoating show that it starts as early as 15 to 30 min post infection and the extent of  
316 uncoating increases strongly until 1 to 2 h post-infection (57, 58). However, the first signs of  
317 replication, including -RNA and +RNA production occur as late as 2 h post-infection (59). This  
318 suggests that the ambient endosomal ion conditions develop gradually to better promote the  
319 uncoating and the final RNA release into the cytoplasm. Current information on the intra-  
320 endosomal ionic changes over time suggests that the sodium and calcium concentrations in  
321 endosomes decrease during their maturation and potassium concentration rises relative to the  
322 extracellular values (39). Our results demonstrated that a combination of low sodium and calcium  
323 and elevated potassium in the presence of physiological magnesium concentration was also able to  
324 trigger the uncoating process, but, however, it was more rapid in the presence of albumin (Fig. 3B  
325 and 3D). Furthermore, in addition to the formation of the uncoating intermediate, the right  
326 combination of ions led to significantly more efficient RNA release (Fig. 2A) that could be further  
327 increased with faf-BSA (Fig. 3E, fluorescence data not shown). In the lack of thorough absolute  
328 ionic concentration measurements inside the endosomes, the present study may underestimate the  
329 complexity of the and actual combination of the ions present. No ions have been resolved in the X-  
330 ray structure of E1, so we cannot directly interpret the effects of the ions on the capsid or the RNA  
331 stability. However, both  $K^+$  and  $Mg^{2+}$  ions can stabilize RNA tertiary structures (60). Thus, we  
332 hypothesize that changes in these ions may contribute to the expansion of the RNA, providing an  
333 additional force to promote conformational change in the capsid and eventual release of the RNA in  
334 the endosome. Changes in the  $Ca^{2+}$  and  $Na^+$  ion concentrations probably affect the protein stability,  
335 through electrostatic interactions.  $Ca^{2+}$  has been shown to be important for the capsid stability of  
336 many viruses (24, 40-44).

337 The RNA-capsid interactions were resolved in structures of A-particles published for several  
338 enteroviruses like enterovirus 71, coxsackievirus A16 and rhinovirus 2, suggesting their importance

339 in assisting RNA release (20, 30, 31). The genome is unique within the capsid, nevertheless, in some  
340 picornaviruses the RNA is so well ordered, that individual bases can be identified ranging from one  
341 base to several (61-66). Here, the icosahedral reconstructions also showed for the first-time details of  
342 the RNA, though not resolved to the atomic level, inside the intact E1 particle and in the uncoating  
343 intermediate structure. The RNA has a high density in proximity to the intact capsid, which  
344 suggests high occupancy, and the probable interaction of the RNA with both the ordered capsid  
345 proteins (notably with VP1, VP4 and VP2 shown in Fig. 5A) and their disordered termini (67).  
346 Trp38 in VP2, highly conserved residue in picornaviruses, points towards ordered RNA (61-66).  
347 This interaction between the RNA and capsid is directly adjacent to the major fenestrations that  
348 appear in the treated capsid, where the RNA has moved radially outwards, maintaining this specific  
349 interaction (Fig. 5D). Interestingly, this RNA-capsid interaction is in proximity to the N-terminus of  
350 VP1 that appears to be extruded from the treated capsid, similarly to the VP1 in expanded  
351 poliovirus, coxsackievirus A16 and enterovirus D68 (20, 26, 49). VP4 could not be identified in the  
352 reconstruction, suggesting that it is no longer icosahedrally-ordered or the occupancy is much  
353 reduced as suggested also by autoradiography. This implies that the RNA interaction with the  
354 capsid via VP4 under the vertices has significantly altered compared to the intact particle (Fig. 5).  
355 VP4 may have collapsed into the density attributed to RNA that is still prominent below the VP3  
356 annulus at the vertices (Fig. 4 and 5). Changes in the RNA packaging density and its interaction  
357 with the capsid proteins, along with the expansion of the particle are probably also responsible for  
358 the increase in permeability to dye.

359 Altogether, our results suggest that, based on the dynamic nature of albumin-mediated fatty acid  
360 binding, and the fact that both albumin and fatty acids are present outside cells, the majority of the  
361 enterovirus particles may reside in a triggered, intermediate, metastable state before entering cells.  
362 According to our results, the albumin-triggered intermediate state is likely to lead to more efficient

363 RNA release when it is further affected by the ambient concentrations of monovalent and divalent  
364 cations in endosomes.

## 365 **Materials and methods**

### 366 **E1 production and purification**

367 E1 was produced and purified as described earlier (14). Confluent 5-layer bottles of green monkey  
368 kidney (GMK) cells, obtained from the American Type Culture Collection (ATCC), were infected  
369 with E1 (Farouk strain, ATCC), for 16-20 hours at 37 °C, 5% CO<sub>2</sub>. After infection, the cells and  
370 media were collected and lysed with three freeze-thaw cycles. The lysate was pelleted by  
371 centrifugation with a JA-10 rotor (6080 rpm, 30 min, +4 °C) after which the supernatant was  
372 precipitated for 16-20 h at + 4°C using polyethylene glycol 6000 (Sigma Aldrich, Saint Louis,  
373 Missouri, US) (8% wt/vol) and NaCl (2.2% wt/vol). The precipitated supernatant was then  
374 centrifuged with a JA-10 rotor (8000 rpm, 45 min, +4 °C) and the resulting pellet was dissolved  
375 into R buffer (10 mM Tris-HCl (pH 7.5), 200 mM MgCl<sub>2</sub>, 10 % (wt/vol) glycerol). For disrupting  
376 the remaining cellular membranes, 0.3 % (wt/vol) sodium deoxycholate (Sigma Aldrich) and 0.6%  
377 (vol/vol) Nonidet P-40 (Sigma Aldrich) were mixed with the supernatant and incubated for 30 min  
378 on ice. The remaining debris was pelleted by centrifuging in a TX-200 rotor (4700 rpm, 15 min, +  
379 4°C), and the resulting supernatant was loaded on top of 10 ml linear 10-40% (wt/volume) sucrose  
380 gradients in R buffer. The gradients were ultracentrifuged in an SW-41 rotor (30000 rpm, 3 h, +  
381 4°C) and fractioned into 500 µl aliquots. The optical density at 260 nm of each fraction was  
382 measured with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts,  
383 US), to identify fractions containing virus. These fractions were dialyzed against 2 mM MgCl<sub>2</sub>-  
384 phosphate buffered saline (PBS-MgCl<sub>2</sub>) in a Spectra/Por Micro Float-A-Lyzer with Biotech  
385 cellulose ester membranes and 300 kDa cut off (Spectrum Laboratories Inc. USA) at +4 °C. Finally,  
386 the viruses were pelleted by ultracentrifugation using a 70Ti rotor (35000 rpm, 2 h, +4 °C) and

387 resuspended in PBS-MgCl<sub>2</sub>. Several purified virus batches were used ranging between 0.5-1.3  
388 mg/ml and  $1 \times 10^{11}$ - $1 \times 10^{12}$  pfu/ml.

#### 389 **<sup>35</sup>S E1 production and purification**

390 <sup>35</sup>S labelled E1 for gradient analysis was produced in GMK cells as described earlier (14) . Semi-  
391 confluent cell culture bottles were washed at 37 °C for 15 min with PBS and infected with E1  
392 (Farouk strain; ATCC) using low-methionine-cysteine medium supplemented with 1% FBS for  
393 three hours. After this the media was changed into the above-mentioned media supplemented with  
394 50 µCi/ml of [<sup>35</sup>S]methionine-cysteine and the infection was allowed to proceed 16-24 h until  
395 nearly all of the cells were detached. The cells were lysed via 3 freeze-thaw cycles and the cell  
396 debris was pelleted using a TX-200 rotor (4000 rpm for 15 min, +4°C). The supernatant was further  
397 treated with 0.3% (wt/vol) sodium deoxycholate and 0.6 % (vol/vol) Nonidet P-40 for 30 min on ice  
398 and the supernatant was further cleared by centrifuging (TX-200, 4700 rpm, 15min, +4 °C). The  
399 supernatant was loaded onto the top of a 2ml 40 % sucrose cushion at +4 °C. The cushions were  
400 ultracentrifuged at 30000 rpm (SW-41) for 2.5 hours at +4 °C after which the liquid above the  
401 cushion and one 500 µl fraction from the cushion were discarded and the three next 500 µl fractions  
402 were collected. These fractions were diluted into PBS-2mM MgCl<sub>2</sub> and the virus was pelleted by  
403 centrifuging 35000 rpm (Ti70) 2-3 h at +4 °C, after which the pellet was resuspended into 1.5 ml of  
404 PBS-MgCl<sub>2</sub> and the different virus populations were further separated in a 5-20% sucrose gradient  
405 in R-buffer via gradient centrifugation (35000 rpm, SW41, 2 h, +4°C). The virus-containing  
406 fractions were identified via liquid scintillation counting (Perkin Elmer, Waltham, Massachusetts,  
407 US). The three fractions containing the highest amount of intact virus particles were again collected  
408 and pelleted as above. The resulted pellet was dissolved into 400 µl of PBS-MgCl<sub>2</sub>, divided into  
409 smaller aliquots and stored at -80°C after determination of CPM/µl of the <sup>35</sup>S E1 stock.

#### 410 **CVB3 and CVA9 production and purification**

411 CVB3 (ATCC, Nancy strain) and CVA9 (ATCC, Griggs strain) were purified in a similarly to E1,  
412 except it was separated in 5-20% sucrose gradient. From the gradient, fractions 1-10 were discarded  
413 and 11-20 collected. The collected fractions were diluted with PBS-MgCl<sub>2</sub> into a final volume of  
414 25ml and concentrated as described for E1.

#### 415 **End-point dilution assay**

416 The end-point dilution infection assay was done as described earlier (68). Shortly, GMK cells were  
417 cultured in 10% serum-containing MEM and infected with E1 in a dilution series in the presence of  
418 1% serum. Typically, after 3 days of infection at +37 °C the 96-well plate was stained with 50 µl of  
419 crystal violet stain (8.3 mM crystal violet, 45 mM CaCl<sub>2</sub>, 10% ethanol, 18.5% formalin and 35 mM  
420 Tris base) for 10 minutes. TCID<sub>50</sub> was determined by calculating the numbers of infected and  
421 uninfected wells for the eight replicates in one 96-well plate at each dilution. Pfu/ml was calculated  
422 by multiplying the TCID<sub>50</sub>/ml value with 0.7 according to the Poisson distribution estimation.

#### 423 **Real-time fluorescence uncoating measurements**

424 The measurements were done using a PerkinElmer 2030 Multilabel Reader Victor X4 with F485  
425 lamp filter and F535 emission filter and one second counting time. In each measurement 1 µg of  
426 non-labelled virus (corresponding to 0.77-2 µl of PBS-MgCl<sub>2</sub>, depending on the stock  
427 concentration) was treated in 100 µl of the buffer described in each experiment in a single well of  
428 96 well plate (Sarstedt, Nümbrecht, Germany) in the presence of 10X SYBR Green II fluorescent  
429 dye. The composition of the buffers is presented in table 1. All of the buffers were neutral in pH  
430 ranging between 7.18-7.44. Where indicated, RNase A was added to the wells at a final  
431 concentration of 10 µg ml<sup>-1</sup>. By adding RNase to the assay, we distinguished between the  
432 fluorescence originating from the porous intermediate particles i.e. from RNA inside the virus  
433 capsid (protected from the RNase activity), and the fluorescence of RNA released from the  
434 particles (sensitive to RNase treatment) indicating the presence of empty particles. Intact virion is  
435 inaccessible to the dye and thus gives a low fluorescence signal (14). For each virus treatment a

436 corresponding blank well with all other factors except virus was also measured and the fluorescence  
437 was subtracted to eliminate fluorescence originating from other factors than the virus. Each well  
438 was measured either every minute or every other minute for a three-hour time period at 37 °C if not  
439 otherwise stated. The plate was prepared on ice, from where it was placed into a preheated  
440 measurement chamber. The results were processed and plotted using Microsoft excel. The error  
441 bars represented the standard error of the mean from a minimum of five technical replicates from at  
442 least two separate biological replicates. In all graphs the amount of fluorescence is normalized to  
443 the end fluorescence value of the control, three-hour DPBS treatment.

#### 444 **Gradient analysis**

445 1 µl (approximately 10,000-80,000 CPM) of 35S E1 together with 1 µg of non-radioactive E1 was  
446 treated for 1 h at +37 °C in 100 µl of the relevant buffer. The samples were then cooled on ice and  
447 loaded on to a cooled 10-ml linear 5 to 20% sucrose gradient. The gradients were centrifuged using  
448 an SW-41 rotor (35,000 rpm, 2 h, +4°C) and 500 µl fractions were collected and mixed with  
449 scintillation cocktail (Ultima Gold MW, Perkin Elmer). The samples were analyzed using Tri-Carb  
450 2910 TR (Perkin Elmer) and plotted in excel. Every treatment was repeated at least once and similar  
451 effects were observed.

452 In the albumin-virus-fatty acid ratio experiment, the molarity of E1 was calculated using molecular  
453 weight estimation of 8 MDa for E1 which resulted in 1.25 nM E1 solution with 1 µg of virus in  
454 100 µl of buffer. According to this, 1200 times higher faf-BSA (Sigma Aldrich, in ddH<sub>2</sub>O)  
455 concentration (1.5 µM, 1200 albumins per virus) of faf-BSA was used for 20:1 ratio of albumin  
456 molecules to virus pocket factors and 120 times higher concentration for 2:1 ratio. 17 times higher  
457 concentration was used for 2:1 ratio between the albumin's high to medium affinity fatty acid  
458 binding sites (7 per albumin) vs. virus pocket factors (60 per virus). Palmitate (Sigma Aldrich) from  
459 15.2 mM stock in 50% MeOH was added in either 20:1 or 50:1 ratio into the 1.5 µM faf-BSA  
460 solution.

461 **EM sample preparation, imaging, particle processing and model building**

462 Virus sample for cryoEM and negative staining was prepared as follows. Purified E1 in PBS-MgCl<sub>2</sub>  
463 buffer at a concentration of 1.27 µg/µl was mixed with 20 mM NaCl, 30 mM K<sup>+</sup> and 0.01 % faf-  
464 BSA buffer giving a final concentration of 0.1 µg/ul virus, 29 mM NaCl, 28 mM K<sup>+</sup>, 0.145 mM  
465 MgCl<sub>2</sub> and 0.0093% faf-BSA (EM buffer) and incubated for 1 h at 37 °C. The uncoating  
466 intermediate formation was confirmed by spectroscopy measurement (Fig. 3E). Control samples  
467 were prepared from purified E1 in PBS-MgCl<sub>2</sub>.

468 Butvar-coated copper grids were glow discharged (EMS/SC7620 Mini sputter coater), after which  
469 the virus sample was added and incubated for 15 s. The excess virus was blotted away and the grids  
470 were negatively-stained using phosphotungstic acid (H<sub>2</sub>O, pH 7.4) for 60 seconds and the excess  
471 stain was blotted away. The samples were stored overnight at RT before imaging with a JEM-1400  
472 (JEOL) transmission electron microscope (80kV, Olympus SIS Quemesa bottom-mounted 11  
473 Megapixel CCD camera, 12,000-40,000 x magnification).

474 Sample volumes of 3 µl of control or EM buffer-treated E1 particle were applied to glow-  
475 discharged Quantifoil holey carbon R2/2 grids and vitrified using a custom-made manual plunger.  
476 The vitrified samples were imaged on a 200 kV Talos Arctica microscope equipped with a Falcon  
477 III direct electron detector at a nominal magnification of 120,000 x corresponding to a calibrated  
478 pixel size of 1.24 Å. Each exposure was 47.8 s long and collected as a movie containing 30 frames  
479 with an accumulated dose of 30 e/Å<sup>2</sup> using Thermo Fisher Scientific's automatic data acquisition  
480 software.

481 Dose-fractionated image stacks were aligned using MotionCor2 (69). The contrast transfer function  
482 parameters for each micrograph were estimated using Gctf (70). Images containing drift or  
483 astigmatism were discarded. Particle selection, 2D classification, initial model generation and 3D  
484 classification were performed using RELION 2.1 (71). Final 3D refinement followed by sharpening  
485 (B-factor -70 Å<sup>2</sup>) of 45309 intact particles from control sample (~45% of total particles) resulted in

486 a 3.5 Å resolution map. After applying the same procedure to 14615 uncoating intermediate  
487 particles from treated sample (~59% of total particles), a 3.6 Å resolution map was generated.

#### 488 **Model building**

489 A crystal structure of the E1 virion (PDB ID: 1EV1) served as an initial starting model for the intact  
490 E1 particle (48). An initial atomic model for the uncoating intermediate particle was generated using  
491 the I-TASSER server (72) based on the crystal structure of the CVA16 uncoating intermediate (PDB  
492 ID: 4JGY) (20, 73). The atomic coordinates of the initial models were docked manually into the  
493 electron density maps using UCSF Chimera and further optimized using the ‘Fit in Map’ command  
494 (74). In the case of the intact particle, one residue (Cys7) was added to the VP2 N-terminus of 1EV1  
495 model using Coot 0.8.8 and the fit was further optimized using molecular dynamics flexible fitting  
496 (MDFF) software used along with NAMD and VMD (69-72) (75). A scale factor of 1 was used to  
497 weigh the contribution of the cryo-EM map to the overall potential function used in MDFF.  
498 Simulations included 10,000 steps of minimization and 100,000 steps of molecular dynamics under  
499 implicit solvent conditions with secondary structure restraints in place. The atomic model of the  
500 uncoating intermediate particle was refined in Coot 0.8.8 and this served as an input for MDFF,  
501 NAMD and VMD software (75-78). Simulations with scale factor of 1 included 20,000 steps of  
502 minimization and 100,000 steps of molecular dynamics under implicit solvent conditions with  
503 secondary structure restraints in place. To minimize atom clashes in the atomic model processed in  
504 MDFF, seven asymmetric units were simultaneously refined in MDFF using the same secondary  
505 structure restraints as above. To analyse unassigned density in intact or treated virion  
506 reconstruction, the atomic model was converted to 3.5 (intact) or 3.6 (treated) Å resolution electron  
507 density map using the ‘molmap’ command in UCSF Chimera. The map generated for the accounted  
508 density was then subtracted from the reconstruction using the ‘vop subtract’ command in UCSF  
509 Chimera. The treated particle expansion was estimated by measuring the particle diameter between  
510 the five-fold vertices in intact and treated virions.

### 511 **Gel separation of viral proteins and autoradiography**

512 1 µg of non-radioactive E1 with 1 µl of <sup>35</sup>S E1 (approximately 10,000-80,000 CPM) was treated for  
513 1 h at 37 °C in EM buffer. Non-treated virus was used as a VP4 detachment negative control and  
514 virus incubated for 10 minutes at 50°C was used as a positive control. Samples were dialysed  
515 against 1 liter of PBS-MgCl<sub>2</sub> for 40 minutes using Slide-A-Lyzer mini dialysis device with 10 kDa  
516 cut off (Thermo Fisher Scientific) to remove the possibly detached VP4 from the samples. The  
517 samples were boiled for 10 min with the sample buffer and ran to 4-20 gradient gel (MINI-  
518 PROTEAN TGX precast gel, Bio-Rad Laboratories, Inc., Hercules, California, US). Precision plus  
519 protein standard 10-250 kDa (Bio-Rad Laboratories, Inc., Hercules, California, US) was used to  
520 distinguish the molecular weight. The gel was fixed in 30% methanol, 10% acetic acid and treated  
521 with autoradiography enhancer (Enlightning, Perkin Elmer). The gel was dried at +70 °C for 2 h  
522 (Gel dryer 583, Bio-Rad Laboratories, Inc., Hercules, California, US) and subjected to  
523 autoradiography. Protein bands were analyzed using ImageJ gel analyzer tool.

### 524 **Data availability**

525 The final density maps have been deposited in the Electron Microscopy Databank (EMDB) with  
526 accession codes EMD-4903 (control E1) and EMD-0565 (expanded E1). The atomic models have  
527 been deposited in the Worldwide Protein Databank (wwPDB) with accession codes 6RJF (control  
528 E1) and 6O06 (expanded E1). The raw data have been deposited in the EMPIAR database with  
529 deposition ID 414.

530

531

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542 validated the data. V.R. and A.D visualized the data. V.R. M.L., A.D, S.J.B., and V.M. wrote the  
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776

## 777 **Figure legends**

778

779 Fig. 1. Echovirus 1 stability. (A) Infectivity of E1 after 1, 5, 15 or 21 days in PBS at +37 °C or  
780 room temperature. (B) Fluorescence measurement of SGII-dye in the presence of 1 µg E1 in PBS-  
781 MgCl<sub>2</sub> (black line) and DPBS (red line) with (dotted line) or without (solid line) RNase during 3 h  
782 at 37 °C. (C) 5-20% sucrose gradient separation of <sup>35</sup>S-labeled E1 when treated for 1 h at +37 °C in  
783 PBS-MgCl<sub>2</sub> (blue) vs. non-treated virus (black). The amount of different virus populations is shown  
784 in the inset. (D) Fluorescence measurement of SGII-dye in the presence of 1 µg E1 in 1% S-MEM  
785 at 37 °C (Black lines). In gray, as an example, results of the same virus measurements (solid line)  
786 before subtracting the blank measurements (dotted line). In the presented results of 1% S-MEM  
787 (black line), and in all of the presented fluorescent results in this paper, the blank, which contains  
788 all other factors except the virus (gray dotted line), is subtracted from the measurement with virus  
789 (gray solid line). (E) 5-20% sucrose gradient separation of <sup>35</sup>S-labeled E1 when treated for 0-60 min  
790 at +37 °C in 1% S-MEM. The amount of different virus populations is shown in the inset. (F)  
791 Fluorescence measurement of SGII-dye in the presence of 1 µg of CVB3 or CVA9 in 1% MEM at

792 +37 °C during 3 h. (G) Fluorescence measurement of SGII-dye in the presence of 1 µg E1 in 1% S-  
793 MEM at room temperature (23 °C) for 60 minutes after which the multilabel reader heater was  
794 turned to 37 °C for 90 minutes. The transition from room temperature to 37 °C lasted about 12  
795 minutes.

796 In all of the fluorescence measurements, in this and other figures, the Y-axis is normalized to the  
797 final fluorescence value of the control DPBS treatment (Fig. 1B, red solid line). Treatments in each  
798 buffer with and without RNase are marked with same colour but dotted and solid lines,  
799 respectively. All fluorescence measurements are averages of minimum five measurements and the  
800 presented error bars are ±standard error of the mean.

801

802 Fig. 2. Virus stability in NK solution with different amounts of  $K^+$ ,  $Mg^{2+}$  (M) and  $Ca^{2+}$  (C) at 37 °C.

803 (A) Fluorescence measurement of SGII-dye in the presence of 1 µg E1 in NKMC, containing 30  
804 mM K phosphate (black lines), NKMC with higher, 60 mM K phosphate (red lines) and NK (i.e.  
805 without  $Mg^{2+}$  and  $Ca^{2+}$ , blue lines) solutions. (B) Fluorescence measurement of SGII-dye in the  
806 presence of 1 µg E1 in NK solution with different amounts of  $CaCl_2$  (C = 200 µM, 0.1C = 20 µM,  
807 0.01C = 2 µM). (C) Fluorescence measurement of SGII-dye in the presence of 1 µg E1 in NK  
808 solution with different amounts of  $MgCl_2$  (M = 500 µM, 0.1M = 50 µM, 0.01M = 5 µM). D)  
809 Fluorescence measurement of SGII-dye at 37 °C in the presence of 1 µg E1 in either intra- and  
810 extracellular  $Na^+$  and  $K^+$  concentrations, combined with intracellular 800 µM  $MgCl_2$  and 0.2 µM  
811  $CaCl_2$  concentrations. (E-F) Fluorescence measurement of SGII-dye at +37 °C in the presence of 1  
812 µg E1 in varying  $Na^+$  and  $K^+$  concentrations between intra- and extracellular values, but constant  
813 intracellular 800 µM  $MgCl_2$  and 0.2 µM  $CaCl_2$  concentrations.

814

815

816 Fig. 3. Serum and albumin promote virus opening at 37 °C. (A) Fluorescence measurement of SGII-  
817 dye in the presence of 1 µg E1 in MEM and 1% S-MEM. (B) Fluorescence measurement of SGII-  
818 dye in the presence of 1 µg E1 in NKMC and 1% S-NKMC. (C) Fluorescence measurement of  
819 SGII-dye in the presence of 1 µg E1 in MEM supplemented with 0.01% BSA or faf-BSA. (D)  
820 Fluorescence measurement of SGII-dye in the presence of 1 µg E1 in NKMC solution  
821 supplemented with 0,01% BSA or faf-BSA. (E) Fluorescence measurement of SGII-dye in the  
822 presence of 1 µg E1 in DPBS supplemented with 0.01% faf-BSA and in EM buffer. (F) Effect of  
823 faf-BSA and fatty acids on virus uncoating and priming using 5-20% sucrose gradient analysis of  
824 metabolically labelled <sup>35</sup>S E1. Non-treated virus (black line) in comparison to fully opened virus  
825 (red line; 1 h incubation with 0.1% faf-BSA NK at 37 °C) and fatty acid-rescued virus after addition  
826 of 400 µM palmitate (blue). (G) Virus treated for 1 h at 37 °C in DPBS (black line) in comparison  
827 to DPBS supplemented with 1.5 µM faf-BSA (20:1 ratio of faf-BSA to virus hydrophobic pockets,  
828 red line), or, in addition to 1.5 µM faf-BSA, with increasing (20:1 and 50:1) molar ratio of  
829 palmitate to the faf-BSA (light blue and dark blue lines, respectively).

830

831 Fig. 4. Electron Microscopy and Cryo-EM reconstructions of E1 intact and treated virions.

832 (A) Negative stain of E1 after 1 h treatment at 37 °C with 29 mM NaCl, 28 mM K, 0.145 mM  
833 MgCl<sub>2</sub>, 0.0093% faf-BSA. (B) Cryo-electron micrographs of non-treated (control) E1 and E1 after  
834 similar treatment to (A). (A & B) The white arrow indicates intact virion, the red arrow indicates  
835 empty, and the green arrow indicates intermediate particle. Scale bar is 50 nm. (C) Example of 2D  
836 class averages showing intact (control, n=16298), treated (treated, n=2015) and empty class  
837 averages (n=212), where n is the number of particles contributing to the average shown. Box size is  
838 496 Å (D) Fourier shell correlation curves for the control E1 reconstruction, giving a resolution of  
839 3.5 Å with a cutoff of 0.143. (E) Fourier shell correlation curves for the treated particle  
840 reconstruction giving a resolution of 3.6 Å with a cutoff of 0.143. (D &E) Corrected (black),

841 unmasked (green), masked (blue) and phase randomized masked (red) maps (71). (F) Control E1  
842 reconstruction central plane. Pentagon, triangle, and oval indicate five-fold, three-fold and two-fold  
843 symmetry axes, respectively. (G) Radially coloured isosurface representation of the control E1  
844 reconstruction viewed down a two-fold axis of symmetry at 1.5 SD above the mean. Pentagons and  
845 triangle mark five-fold and three-fold axes, respectively. Two-fold axis is in the middle between the  
846 two marked five-fold axes. (H) Control E1 coloured by local resolution (79). (I) Treated particle  
847 reconstruction central plane. (J) Isosurface representation of treated particle viewed down a two-  
848 fold axis of symmetry at 1.5 SD above the mean. (G & J) Radially coloured according to the colour  
849 key in (G). (K) Treated particle coloured by local resolution. (J & K) Resolution coloured according  
850 to the colour key in (H).

851

852 Fig. 5. Comparison of reconstructions and atomic models of control and treated E1 virions. (A) Slab  
853 of the virion atomic model (1EV1) (48) shown in ribbon, fitted into the control E1 density on the  
854 left-hand side. Most of the capsid density is accounted for by the atomic model, but the inner  
855 density from the RNA is not. The upper right inset highlights the structure of one pentamer with  
856 VP4. The lower right inset highlights the interaction of the RNA and VP2 Trp38 next to the two-  
857 fold. (B) Section of control E1 reconstruction revealing the pocket and the lipid factor. (C) The  
858 same as in (B) with the atomic model fitted. (D) Slab of the treated atomic model (wwPDB  
859 deposition ID 6O06) shown in ribbon, fitted into the treated particle density on the left-hand side.  
860 Most of the capsid density is accounted for by the atomic model, but the inner density from the  
861 RNA is not. The position of the RNA has moved radially outwards as the capsid expanded,  
862 maintaining the two-fold connections. The upper right inset highlights the structure of one pentamer  
863 without VP4 modelled as there was no apparent density for it. The lower right inset highlights the  
864 interaction of the RNA and VP2 Trp38 next to the two-fold. (E) Section of treated particle  
865 reconstruction revealing the collapsed pocket and no evident density for the lipid factor. (F) The

866 same as in (E) with the atomic model fitted. (A-F) EM density shown in transparent grey, lipid  
867 factor EM density in orange, VP1 (blue ribbon), VP2 (cyan ribbon), VP3 (green ribbon), VP4  
868 (yellow ribbon), lipid factor (orange stick). (B & E) Orange oval indicates the corresponding  
869 positions of the pocket.

870

871 Fig. 6. Treated particle expansion and unmodelled cryo-EM density. (A) Atomic model of control  
872 E1 seen along two-fold axis. (B) Atomic model of the treated intermediate virion seen along two-  
873 fold axis showing opening between two VP2 helices. (C) Unmodelled cryo-EM density of control  
874 E1 (grey isosurface). Inset shows unmodelled density at the three-fold axis. Red arrows indicate N-  
875 termini of VP2 (Cys 7) in the vicinity of unmodelled density blob. Triangle indicates three-fold  
876 symmetry axis. (D) Unmodelled cryo-EM density of the treated virion (grey isosurface). Inset  
877 shows unmodelled density spanning the capsid near two-fold axis. Red arrows indicate N-termini of  
878 VP1 (Asn 55) in the vicinity of the unmodelled density spanning the capsid. Pentagon and oval  
879 indicate five-fold and two-fold symmetry axes, respectively. (A-D) VP1 (blue), VP2 (cyan), VP3  
880 (green), VP4 (yellow), lipid factor (orange).

881

882 Fig 7. Autoradiography observation of  $^{35}\text{S}$ -labelled VP4 protein. Metabolically labelled E1, non-  
883 treated, heated to 50°C for 10 minutes, and EM buffer treated sample.

884

885

## Tables

Table 1. Exact compositions of the buffers used in the paper. The concentrations are in mM.

Buffer/ compound	NaCl	Na <sub>2</sub> HPO <sub>4</sub>	Total Na	KCl	KH <sub>2</sub> PO <sub>4</sub>	K <sub>2</sub> HPO <sub>4</sub>	Total K	MgCl <sub>2</sub>	CaCl <sub>2</sub>	faf-BSA	pH
PBS	137	8	145	3	2		5				7.34
PBS-MgCl <sub>2</sub>	137	8	145	3	2		5	2			7.22
DPBS	138	8.1	146.1	2.7	1.5		4.2	0.5	0.9		7.25
NKMC	20		20		6	12	30	0.5	0.2		7.18
NK	20		20		6	12	30			0.0093%	7.25
EM buffer	29		29		5.6	11.2	28	0.145			7.28

Table 2. Summary of cryo-EM data collection, refinement, and validation statistics.

	Treated E1	Control E1
<b>Data collection</b>		
Voltage (kV)	200	200
Electron exposure (e-/Å <sup>2</sup> X s)	30	30
Pixel size (Å)	1.24	1.24
Number of micrographs	1,246	979
<b>Reconstruction</b>		
Number of particles	14615	45309
B factor (Å <sup>2</sup> )	-70	-70
FSC threshold	0.143	0.143
Resolution (Å)	3.6	3.5
<b>Model building</b>		
VP1 (full protein 281 aa)	55 – 130 and 137 – 280	1 – 281
VP2 (full protein 261 aa)	12 – 54 and 58 – 261	7 – 261
VP3 (full protein 239 aa)	3 – 174 and 184 – 237	1 – 239
VP4 (full protein 68 aa)	none	1 – 14 and 22 – 68
<b>Model validation</b>		
MolProbity score	1.25/100 <sup>th</sup> percentile*	1.08/100 <sup>th</sup> percentile**
Ramachandran outliers (%)	4.3	2.06
Poor rotamers (%)	1.7	1.24
Clashscore	0	0

\*(N=342, 3.25Å - 3.85Å); \*\*(N=27675, 0Å - 99Å)













