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1 **CD63 sorts cholesterol into endosomes for storage and distribution via**
2 **exosomes**

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34 **Abstract**

35 Extracellular vesicles (EV) such as exosomes are now recognized as key players of intercellular
36 communication. Their role is influenced by their specific composition in protein and lipid sets
37 that are sorted during their generation as intraluminal vesicles (ILV) in multivesicular
38 endosomes. Here, we report that a key component of small EVs, the tetraspanin CD63, sorts
39 cholesterol to ILVs, generating a pool that can be mobilized by the NPC1/2 complex and
40 exported via exosomes to recipient cells. In absence of CD63, cholesterol is retrieved from
41 endosomes by an actin-dependent vesicular transport, placing CD63 and cholesterol at the
42 center of a balance between inward and outward budding of endomembranes. These results
43 establish CD63 as a lipid sorting mechanism within endosomes and show that ILVs and
44 exosomes are alternative providers of cholesterol.

45

46

47 **Introduction**

48 Within the endosomal pathway, coordination of outward and inward membrane remodeling and
49 sorting mechanisms enable the recycling of materials towards the Golgi complex or the plasma
50 membrane¹⁻³, as well as their packaging into intraluminal vesicles (ILVs) of Multivesicular
51 Endosomes (MVEs)^{4,5}. Efficient sorting to ILVs is vital for various cellular functions such as
52 cell signaling and protein catabolism. The composition of ILVs, including proteins and lipids,
53 influences their function post-secretion as exosomes in the extracellular environment^{6,7}. While
54 sorting mechanisms for proteins have been extensively studied^{4,8}, the mechanisms responsible
55 for enriching specific sets of lipids on ILVs remain poorly understood.

56 CD63 is a member of the tetraspanin family that organize themselves into lipid microdomains
57 enriched in gangliosides, cholesterol, and a network of partner proteins whose trafficking,
58 cleavage, and functions they regulate⁹⁻¹¹. Unlike other tetraspanins, CD63 is primarily localized
59 within late endosomes/MVEs, lysosomes, where it is concentrated in ILVs and hence enriched
60 in exosomes¹², making it a reliable marker and tool to label and manipulate this EV
61 subpopulation^{13,14}. CD63 is involved in diverse cellular processes, including cargo transport
62 and sorting¹⁵. Its roles vary depending on the cell type studied; for instance, it regulates the
63 targeting of CXCR4 to endolysosomes in T cells¹⁶ and the clustering of P-selectin at the plasma
64 membrane of endothelial cells¹⁷. In melanocytes¹⁸ and Epstein–Barr virus (EBV)-infected B
65 cells, CD63 functions as both a cargo^{5,19} and a regulator¹⁸ of sorting mechanisms at MVEs^{18,}
66 ^{20, 21,22}.

67 Here we investigated whether a primary molecular mechanism would underlie CD63's various
68 roles, by comparing the consequences of CD63 depletion in two different cell lines. The
69 findings reveal a fundamental role for CD63 in sorting cholesterol to ILVs and exosomes. By
70 promoting the accumulation of cholesterol within ILVs, CD63 regulates endosomal membrane
71 remodeling. This pool of stored cholesterol can be retrieved by cells from ILVs and exosomes
72 in a manner dependent on NPC1. Thus, CD63 emerges as a key regulator of cholesterol
73 endosomal sorting, providing alternative sources of cholesterol through ILVs and exosomes.

74

75

76 **Results**

77 **CD63 does not affect sEV protein composition and secretion**

78 To identify the shared role of CD63, we compared two commonly used cell lines for CD63
79 research: HeLa²³ and MNT-1^{18,20}. Using CRISPR/CAS9, we generated CD63 knockout (KO)
80 HeLa and MNT-1 cells, obtaining two HeLa KO clones (KO#1 and KO#2) and one CD63 KO
81 MNT-1 clone. Western blot analysis of cell lysates confirmed complete CD63 depletion without
82 notable changes in CD9, Alix (both cell types), syntenin (HeLa), or endogenous ApoE (MNT-
83 1) (Fig. 1a). Analysis of small extracellular vesicles (sEVs) from wild-type (WT) and CD63 KO
84 cells showed no significant differences in sEV markers (Fig. 1b) except for reduced ApoE levels
85 in CD63 KO MNT-1-derived sEVs, as previously reported²⁰.

86 Nanoparticle tracking analysis (NTA) of sEVs obtained by differential ultracentrifugation (UC)
87 (Fig. 1c) or size exclusion chromatography (SEC) (Fig. 1, Extended Data Fig. 1a) demonstrated
88 similar sEV release and size distribution between WT and CD63 KO cells, consistent across
89 different growth media conditions (Extended Data Fig. 1b,c).

90 To mitigate potential compensatory mechanisms induced by long-term tetraspanin depletion²⁴,
91 we performed similar analyses in MNT-1 cells treated with Ctrl siRNA or CD63 siRNA and on
92 sEVs isolated from cells grown in lipoprotein-depleted serum (LPDS)-supplemented media.
93 Partial CD63 depletion by siRNA did not affect cellular and sEV markers or the number and
94 size distribution of sEVs, except for reduced ApoE levels in sEVs (Extended Data Fig. 1d,e).
95 Analysis of endolysosomal compartments revealed comparable patterns between WT and
96 CD63 KO HeLa cells (Fig. 1e,f), in line with unaffected sEV production.

97 We conducted quantitative mass spectrometry analyses of whole cell membranes from WT or
98 CD63 KO HeLa or MNT-1 cells and corresponding sEVs isolated by SEC (Fig. 2 and Extended
99 Data Fig. 2). CD63 depletion affected the level of a very minute number of proteins in HeLa
100 (Extended Data Fig. 2a,b) or MNT-1 (Extended Data Fig. 2c,d) cell lysates, with no prior

101 associations with CD63. GO-Term analysis showed proteins enriched in WT MNT1 compared
102 to KO associated with membrane raft and EVs (Extended Data Fig. 2d). On sEVs, several
103 proteins were decreased upon CD63 depletion in HeLa-derived sEVs, including MMP12 that
104 displays similar expression pattern as CD63²⁵ and the scaffold protein BASP1 that is enriched
105 in EVs thanks to a myristoylated moiety²⁶, while in MNT-1-derived sEVs, VGF secretion is
106 correlated to VAMP7 dependent CD63 secretion²⁷ and LGALS3BP can be enriched in sEV
107 overexpressing GFP-CD63¹³ but dissociates from EV upon sucrose gradient isolation²⁶
108 (Extended Data Fig. 2e-h).

109 Correlation plot analysis of proteins enriched in WT condition compared to CD63 KO in both
110 HeLa and MNT-1 cell lysates identified CD63 as the only significant change, with no specific
111 associated GO-term (Fig. 2a and 2b). In sEVs, none of the previously identified proteins was
112 commonly depleted from both cell types (Fig. 2c and d) and the ones commonly depleted were
113 not linked to the endolysosomal pathway, with the GO-term mainly revealing enrichment in
114 transmembrane transporter activity.

115 In summary, CD63 depletion neither affected sEV release nor induced common changes in sEV
116 protein composition or steady-state level of proteins in both cell types. During the publication
117 process, similar findings were confirmed in HEK293²⁸ and MCF7 cells²⁹.

118

119 **CD63 regulates cellular lipid homeostasis**

120 Despite the lack of a clear effect of CD63 depletion on sEV protein composition or number, we
121 turned our focus to other common EV cargo molecules. The enrichment of soluble proteins
122 associated with membrane microdomains in WT sEVs and the specific affinity of tetraspanins
123 for certain lipids such as cholesterol and gangliosides^{8,9} prompted us to investigate the influence
124 of CD63 expression on lipid homeostasis. We conducted lipidomic analyses using mass
125 spectrometry of whole cell membranes extracted from HeLa and MNT-1 WT or CD63 KO
126 cells, as well as sEVs/exosomes isolated from the same cells by SEC. To prevent the co-
127 isolation of exogenous lipoproteins with sEVs, cells were cultured in LPDS supplemented
128 media for at least two days before collecting the conditioned media and processing the cells for
129 further analyses.

130 Lipidomic analysis of sEVs from both HeLa-WT (Fig. 3a and Extended data Fig. 3a) and MNT-
131 1-WT (Fig. 3b and Extended data Fig. 3b) revealed that sEVs predominantly consisted of
132 phosphatidylcholine (PC), phosphatidylethanolamine (PE), plasmalogen PE (PE-P),
133 sphingomyelin (SM), or free cholesterol (FC), consistent with previous reports in other cell
134 types^{30,31}. Compared to whole cell lysates, sEVs were enriched in FC, cholesteryl esters (CE),

135 SM, and PE-P in HeLa cells, while being depleted of bis(monoacylglycerol)phosphate (BMP),
136 phosphatidylinositol (PI), PC, lysophosphatidylglycerol (LPG), and phosphatidic acid (PA)
137 (Fig. 3a). Similarly, sEVs from MNT-1 WT cells were enriched in FC, CE, SM, and
138 lysophosphatidylcholine (LPC), while being depleted in BMP, PC, PE, PI,
139 phosphatidylglycerol (PG), and LPG (Fig. 3b). Notably, FC, CE, and SM were commonly
140 enriched in sEVs from both cell types, suggesting potentially elusive active sorting processes.
141 In contrast, BMP, enriched on intraluminal vesicles (ILVs) of late endosomes³², and Ceramide
142 (Cer), implicated in ILV formation³³, were not enriched.

143 CD63 knockout resulted in a slight but significant decrease in FC, Cer, and PE-P in HeLa cells
144 (Fig. 3c and Extended data Fig. 3a), and an increase in PE in MNT-1 cells (Fig. 3d and Extended
145 data Fig. 3b). When comparing isolated sEVs, HeLa CD63 KO sEVs exhibited lower levels of
146 LPG compared with HeLa WT sEVs (Fig. 3e and Extended data Fig. 4a), while MNT-1 CD63
147 KO showed increased CE levels and decreased PE-P, BMP, lysophosphatidylethanolamine
148 (LPE), and FC levels (Fig. 3f and Extended data Fig. 4b). Additionally, we analyzed the PC to
149 PE molar ratio (PC/PE) in cells and sEVs as a marker of global changes in lipid homeostasis.
150 The ratio was affected by CD63 depletion in sEVs but not in cells in HeLa, and inversely in
151 MNT-1(Extended data Fig. 4c,d).

152 Overall, lipidomic analyses indicated that upon CD63 depletion, there was a slight modulation
153 of cholesterol homeostasis common to both cell types, suggesting a potential alteration in
154 cholesterol trafficking. As cholesterol directly or indirectly^{9,34,35} interacts with tetraspanins and
155 is co-enriched with CD63 on ILVs³⁶ and sEVs³⁰, we further investigated the role of CD63 in
156 cholesterol homeostasis at the endosomal level.

157

158 **CD63 modulates intracellular cholesterol trafficking.**

159 Cholesterol homeostasis in late endosomes and lysosomes has been primarily studied in the
160 context of its delivery to lysosomes via endocytosis of lipoproteins. In endosomal
161 compartments, Niemann-Pick Type C1 and 2 proteins (NPC1 and NPC2) facilitate the
162 translocation of exogenous cholesterol from lipoproteins to the limiting membrane of late
163 endosomes³⁷. Cholesterol is then transported to other organelles, mainly the endoplasmic
164 reticulum (ER), via non-vesicular trafficking pathways involving membrane contact sites^{37,38}.

165 We initially analyzed the effect of CD63 depletion on the capacity of cells to uptake and process
166 LDL-derived cholesterol. Incubation of WT and CD63 KO HeLa cells with fluorescent LDL
167 (LDL-BODIPY) did not reveal any significant defect in the endocytosis of exogenous
168 cholesterol sources (Fig. 4a). Immunolabeling of cholesterol using the D4 fragment of the

169 cholesterol-binding toxin perfringolysin O (θ toxin, *Clostridium perfringens*) fused to GFP (D4-
170 GFP)^{39,40} and observation by transmission electron microscopy (TEM) showed similar levels
171 of cholesterol staining in multivesicular bodies (MVBs) of HeLa WT and CD63 KO cells grown
172 in the presence of serum-derived LDL-cholesterol (Extended data Fig. 5a). Treatment with
173 U18666A, an inhibitor of the cholesterol transporter NPC1, induced similar accumulation of
174 LDL-BODIPY associated fluorescence in endosomal compartments in both WT and CD63 KO
175 HeLa cells, indicating that the NPC1 pathway was active in both conditions (Fig. 4a).
176 Next, we examined endogenous cholesterol homeostasis upon CD63 depletion by culturing
177 cells in LPDS supplemented media. Global cellular cholesterol concentrations measured using
178 a fluorometric method (Amplex Red) showed no significant decrease (Extended data Fig. 5b).
179 Evaluation of changes in cholesterol homeostasis through SREBP2 cleavage state and nuclear
180 translocation did not reveal significant differences between WT and CD63 KO cells (Extended
181 data Fig. 5c). Imaging of Nile red positive lipid droplets also showed no change in intensity or
182 appearance in CD63 KO cells (Extended data Fig. 5d).
183 Subsequently, we analyzed endogenous cholesterol by labeling cells grown in LPDS
184 supplemented media with various dyes against cholesterol. Treatment with U18666A resulted
185 in a relative enrichment of Filipin or D4-GFP staining in WT cells but not in CD63 KO cells
186 (Fig. 4b,c). We identified CD63 positive structures as the subcellular localization of
187 endogenous cholesterol by immunofluorescence using recombinant D4-GFP or expressing D4-
188 mCherry constructs (Fig. 4d,e), and used TEM to refine cholesterol localization to ILVs of
189 CD63 positive MVEs (Fig. 4f-h). Strikingly, CD63 KO HeLa cells showed no relative
190 enrichment in Filipin staining upon treatment with U18666A (Fig. 4b), and the labeling with
191 recombinant D4-GFP or D4-mCherry constructs was decreased (Fig. 4c,e,f,h). Similar defects
192 in cholesterol sorting to ILVs were observed in MNT-1 CD63 KO and MNT-1 cells treated
193 with CD63 siRNA (Extended data Fig. 5e,f). In WT cells we also observed MVEs completely
194 devoid of cholesterol, supporting the existence of subpopulations of MVEs³⁶. These findings
195 suggested that CD63 generates a transient storage pool of endogenous cholesterol on ILVs that
196 can be retrieved by the NPC1 pathway.

197

198 **CD63 influences vesicular retrograde transport**

199 In the absence of CD63, unlike other cargoes whose sorting to ILVs is compromised^{18,41},
200 cholesterol did not accumulate on the limiting membrane of MVEs (Fig. 4f-h). CD63 depletion
201 does not affect cholesterol accumulation from lipoparticles upon U18666A treatment but from
202 ILVs (Fig. 4a-b). Hence, the most plausible hypothesis is that in the absence of CD63,

203 cholesterol is retrieved from the endosomal limiting membrane before being sorted to ILVs,
204 and is transported to other compartments. SREBP2 cleavage and nuclear translocation were
205 unaffected by CD63 depletion (Extended data Fig. 5c), and our EM analysis excluded the
206 mobilization of membrane contact sites in this phenotype. While cholesterol did not accumulate
207 at the plasma membrane, we observed increased D4-GFP staining at the Golgi apparatus upon
208 CD63 depletion in both cell types (Fig. 4g-h and Extended data Fig. 5f,g).

209 To further track cholesterol trafficking, we fed cells grown in LPDS supplemented media with
210 TopFluor (TF-chol), a fluorescent analog of cholesterol that traffics like cholesterol^{42,43}. TF-
211 chol localized both at the plasma membrane and in perinuclear compartments at similar levels
212 in WT and KO cells but displayed a more perinuclear localization in the KO condition (Fig. 5a-
213 c). In the presence of U18666A, TF-chol intensity increased, TF-chol accumulated in swollen
214 compartments in both conditions, but the number of TF-chol positive compartments was
215 drastically reduced and more perinuclear in CD63 KO cells. In CD63 KO cells, the brightest
216 compartments surrounded an area of weak diffuse fluorescent signal in the perinuclear region
217 that may correspond to the Golgi Apparatus (Fig. 5a). Analysis of U18666A-treated cells
218 showed increased colocalization of TF-chol with a marker of (medial-) Golgi, Mannosidase-II,
219 and decreased colocalization with the late endosomes/lysosomes marker LAMP-1 in the
220 absence of CD63 (Fig. 5d,e). These data suggest that cholesterol accumulates in the Golgi
221 apparatus in the absence of CD63.

222 We then monitored the anterograde and retrograde transport of cargos whose trafficking is
223 influenced by cholesterol levels^{44,45}. Upon addition of biotin, viral glycoprotein VSVG inserted
224 in the RUSH system was able to exit the ER, reach the Golgi, and the plasma membrane in both
225 WT and CD63 KO HeLa cells, confirming that anterograde trafficking pathways are not
226 affected by CD63 absence (Extended data Fig. 6a). Pulse chase experiments revealed an altered
227 redistribution of cation-independent mannose 6-phosphate receptor (CI-M6PR) to the Golgi
228 apparatus in CD63 KO HeLa cells and a similar trend in CD63 siRNA treated MNT-1 cells
229 (Extended data Fig. 6b,c). We then focused on endogenous ApoE, a known regulator of
230 cholesterol homeostasis expressed in MNT-1 cells but not in HeLa cells. In line with the
231 reported role of CD63 in sorting endogenous ApoE to ILVs²⁰, we confirmed that siRNA-
232 mediated depletion of CD63 or CD63 KO led to the accumulation of ApoE in TGN and Golgi
233 and its depletion from LAMP-1 positive lysosomes (Extended data Fig. 6d-f). These data
234 suggest that retrograde but not anterograde transport of cargos sensitive to cholesterol levels is
235 affected in the absence of CD63.

236 We refined our morphological analysis of MVEs in HeLa and MNT-1 cells via TEM. In CD63
237 KO cells, MVEs exhibited increased tubules and budded structures nearby (Fig. 5f). EM
238 analysis after high-pressure freezing of CD63 KO MNT-1 cells also revealed similar tubules
239 and budded structures near endosomes corresponding to early melanosomes¹⁸ (Extended data
240 Fig. 7a) (positive for D4-GFP in WT cells). Given the crucial role of actin bundling in
241 endosomal tubular domain fission for cargo recycling³, we investigated endosome-associated
242 actin distribution using phalloidin staining and SR-IFM. CD63 depletion led to actin
243 accumulation around Lamp-1 positive late endosomes and EEA1 positive early endosomes,
244 which was reversed by CK666, an Arp 2/3 complex inhibitor. Similar actin accumulation was
245 observed in CD63 siRNA treated MNT-1 cells (Fig. 5g and Extended data Fig. 7b,c). Consistent
246 with Arp2/3-dependent actin bundling's role in endosomal tubule fission⁴⁷, TEM analysis of
247 CK666-treated cells showed increased endosomal tubulations in CD63 KO cells (Extended
248 Data Fig. 7d). We then examined whether CK666 affected cholesterol trafficking, revealing
249 altered TF-chol distribution patterns in CD63 KO cells compared to WT cells upon CK666
250 treatment, along with D4-GFP staining accumulation on endosomal tubular carriers in CD63
251 KO cells treated with CK666 (Extended data Fig. 7e,f). These findings suggest that CD63
252 depletion stimulates a retrograde vesicular pathway from endosomes to Golgi, mediated by
253 Arp2/3-dependent actin polymerization, impacting the retrograde transport of CI-M6PR and
254 endogenous ApoE differently.

255

256 **CD63 accommodates cholesterol in an intramembrane cavity**

257 A recent study identifying a cholesterol binding pocket in CD81's crystal structure³⁴ prompted
258 us to construct a 3D model of CD63 WT. The model resembles the secondary topology of
259 CD81, featuring a cone-like structure and an intramembrane cavity (Fig. 6a). Alignment of
260 CD63 sequences with CD81 and CD9 revealed a glutamate residue at position 217 (E217) in
261 CD63's fourth transmembrane domain (Fig. 6b), analogous to E219 in CD81, known for
262 hydrogen bonding to cholesterol hydroxyl groups. Substituting glutamate with glutamine
263 (E217Q) in CD63 was hypothesized to abolish cholesterol binding. We used HADDOCK to
264 model cholesterol interaction with CD63's fourth transmembrane domain. The resulting models
265 showed cholesterol accommodation in the intramembrane cavity of CD63 WT, whereas in
266 CD63 E217Q, cholesterol preferred the cytoplasmic leaflet outside the cavity (Fig. 6c).
267 Expressing WT and E217Q mutant in CD63 KO cells (Fig. 6d) demonstrated correct sorting to
268 sEVs and ILVs (Fig. 6d,e). Filipin staining of LPDS-grown cells showed significant cholesterol
269 accumulation with CD63 WT or E217Q, unaffected by U18666A (Fig. 6f). However, D4-GFP

270 and D4-mCherry staining revealed reduced cholesterol levels in CD63 E217Q upon U18666A
271 treatment compared to CD63 WT (Fig. 6g,h and Extended Data Fig. 8a). These findings
272 confirm CD63's role in cholesterol endosomal trafficking, associated with its ability to
273 accommodate cholesterol in an intramembrane cavity.

274

275 **CD63 regulates cholesterol trafficking to sEVs of endosomal origin**

276 Impaired cholesterol sorting on ILVs of CD63 KO cells prompted us to investigate its
277 relevance for ILVs secreted as exosomes. Initially, we quantified global cholesterol content in
278 sEVs using Amplex Red assay. Cholesterol levels varied considerably in WT cells and tended
279 to decrease in CD63 KO, though not significantly (Extended Data Fig. 8b). To refine this, we
280 performed single vesicle analysis using EM immunogold labeling of cholesterol with D4-GFP.
281 The labeling clustered, possibly representing cholesterol-enriched microdomains, consistent
282 with lipid rafts⁴⁸ and tetraspanin microdomains⁴⁹ on vesicle surfaces (Fig. 7a,c, white
283 arrowheads). sEVs from both HeLa and MNT1 cells grown in LPDS showed reduced D4-GFP
284 labeling upon CD63 KO (average 2.6 PAG/vesicles for WT vs. 1.4 PAG/vesicles for CD63 KO
285 for HeLa sEVs). Notably, the fraction of sEVs with low cholesterol staining (0-1 PAG/vesicles)
286 doubled for CD63 KO EVs, while the fraction with high cholesterol staining (>4 PAG/vesicle)
287 decreased (Extended Data Fig. 8c,d). Re-expressing CD63 WT or CD63 E217Q in HeLa CD63
288 KO confirmed CD63's role in cholesterol sorting to sEVs (Fig. 6d, e). Though sEV numbers
289 remained unchanged (Extended Data Fig. 8e), D4-GFP staining on sEVs was rescued with
290 CD63 WT but not CD63 E217Q (Fig. 7a,b). This aligned with reduced D4-GFP labeling on
291 ILVs from CD63 E217Q-rescued cells compared to CD63 WT-rescued cells (Extended Data
292 Fig. 8f).

293 The discrepancy between Amplex Red assay and other dyes can be attributed to factors such as
294 sEV subpopulation heterogeneity, D4 and Filipin sensitivity to cholesterol esterification⁵⁰, their
295 detection threshold at 30 mol% revealing only cholesterol-rich microdomains^{51,52}, the detection
296 of GM1 by Filipin⁵³. Treating cells with Bafilomycin-A1 enriched sEVs of endosomal origin
297 (Extended Data Fig. 8g), revealing a significant decrease in cholesterol content in sEVs from
298 CD63 KO cells (Fig. 7d). Samples not treated with esterase yielded similar results (Fig. 7d),
299 excluding a role for CD63 in cholesteryl ester sorting.

300

301 **CD63 influences the biophysical properties of sEVs**

302 Changes of the lipid content, particularly cholesterol, of membrane leaflets influences their
303 physical properties. To quantitatively assess the impact of cholesterol depletion on the

304 mechanical properties of ILVs and corresponding sEVs, we employed atomic force microscopy
305 (AFM) nanoindentation. Overview images were obtained with imaging forces <100 pN to
306 determine particle size distribution (Fig. 8e) Upon substrate adhesion, the flexibility of sEVs
307 caused them to deform into various shapes, including spherical caps (Fig. 8f). Determination
308 of the radius of curvature (R_c) of adhered sEVs by cross-sectional analysis revealed that the
309 calculated radius (R_0) of particles in solution was similar between CD63 WT and KO sEVs
310 (Fig. 8g), consistent with NTA analysis (Fig. 1c). Indenting the center of the sEVs with the
311 AFM tip enabled measurement of vesicle membrane stiffness (k) and determination of
312 membrane tension and tether forces (Fig. 8h). Stiffness of HeLa WT sEVs (0.010 ± 0.002 N/m)
313 and HeLa CD63 KO sEVs (0.010 ± 0.001 N/m) showed no significant difference. However,
314 fitting this data to a Canham-Helfrich-theory based model to derive the bending modulus, an
315 intrinsic membrane property, yielded 11 ± 3 kBT for HeLa WT sEVs and 14 ± 4 kBT for HeLa
316 CD63 KO sEVs, with a statistically significant difference ($p = 0.05$). (Fig. 8i).

317 Cholesterol, known as a cryoprotectant, could influence sEV storage. We examined the effect
318 of sEV storage at -80°C on the bending modulus. Addition of data from frozen samples did not
319 significantly alter the bending modulus of HeLa WT sEVs (12 ± 4 kBT (S.E.M., $N=49$)).
320 Conversely, for HeLa CD63 KO sEVs, the bending modulus significantly increased to (19 ± 5
321 kBT (S.E.M., $N=39$), $p = 0.05$) with the addition of frozen data, indicating a direct impact of
322 CD63 absence on sEV mechanical characteristics potentially exacerbated by specific storage
323 conditions.

324 These findings suggest that CD63 KO may mechanically affect sEV membrane stiffness,
325 correlating with cholesterol depletion.

326

327 **CD63 sEVs facilitate cholesterol transfer to recipient cells**

328 The lipid composition and biophysical properties of sEVs are believed to influence their ability
329 to interact with and be internalized by recipient cells. To assess uptake by recipient HeLa cells,
330 we employed a dual tagging approach to load sEVs with TF-chol and/or label them with the
331 membrane dye MemBright⁶⁰ (Extended Data Fig. 9a), which were then fed to unlabeled cells.
332 Initially, we quantified absolute uptake levels by monitoring the accumulation of MemBright-
333 associated sEVs (Fig. 8a,b). Recipient HeLa WT or CD63 KO cells exhibited similar efficiency
334 in capturing sEVs from both HeLa WT and CD63 KO cells. As recipient cell interaction
335 remained unaffected, we investigated whether sEVs could supply cholesterol to recipient cells
336 akin to lipoproteins. Co-tracking of TF-chol and MemBright revealed that both tags
337 accumulated in the same compartments within WT or CD63 KO recipient cells (Fig. 8b).

338 Subsequent treatment with the NPC1 inhibitor U18666A led to TF-chol accumulation in
339 endosomal compartments of LPDS-grown recipient cells, regardless of CD63 status (Fig. 8b,c),
340 indicating that sEVs can provide cholesterol to recipient cells through the action of NPC1/2 in
341 host endolysosomes.

342 Consistent with CD63's role in cholesterol enrichment on sEVs, feeding WT or CD63 KO
343 recipient cells with equivalent CD63 KO sEVs resulted in decreased cholesterol accumulation
344 (Fig. 8c) but not MemBright (Fig. 8a and Extended Data Fig. 9b) compared to WT sEVs. This
345 cholesterol supply deficiency was exacerbated following U18666A treatment (Fig. 8c). Under
346 these conditions, minimal cholesterol accumulated in WT or CD63 KO cells when fed CD63
347 KO-derived sEVs (Fig. 8c and Extended Data Fig. 9c). These findings suggest that CD63
348 depletion and cholesterol removal from EVs do not impede their uptake by recipient cells but
349 impair their ability to act as an external cholesterol source.

350

351 **Discussion**

352 The work identifies CD63 as a pivotal regulator of cholesterol sorting to ILVs and extracellular
353 vesicles across different cell types. In the absence of CD63, impaired cholesterol sorting is
354 compensated by its retrieval from endosomes via an actin-dependent vesicular pathway.
355 Although CD63 and cholesterol depletion do not profoundly affect sEV composition or
356 secretion, they modulate their physical characteristics. Importantly, the cholesterol sorted to
357 ILVs forms a transient storage pool retrievable by the NPC1/2 machinery either from their
358 origin compartment or upon ILV secretion as exosomes within recipient cell endolysosomes.

359 This study establishes CD63-dependent cholesterol sorting as the primary mechanism
360 supporting the active enrichment of a lipid to ILVs and consequently to EVs likely
361 corresponding to exosomes. Only a few lipids, including SM, FC, and CE, are enriched in EVs
362 compared to whole cells, indicating their active sorting on ILVs or plasma membrane-derived
363 buds. CD63 depletion or mutation uniquely impacts free cholesterol concentration, as observed
364 through various approaches in both cell types. Other lipids like BMP are enriched on ILVs via
365 an Alix-dependent mechanism⁶¹ but are poorly present on EVs, suggesting that BMP-enriched
366 ILVs are unlikely to be secreted. This supports the hypothesis that cholesterol-enriched MVE
367 subpopulations^{36,62}, but not BMP-enriched ones, are preferentially secreted.

368 Tetraspanins, including CD63, organize as dynamic nanoclusters in membranes. While early
369 studies highlighted the role of lipids in tetraspanin-enriched microdomain organization^{9,10}, it is
370 increasingly clear that tetraspanins can also influence specific lipid presence in membranes,
371 notably cholesterol and oxysterol. This influence may occur through tetraspanin

372 palmitoylation⁹ or cholesterol recognition/interaction amino-acid consensus (CRAC)
373 sequences, present in most human tetraspanins including CD63³⁵. Alternatively, cholesterol
374 could be accommodated in an intramembrane cavity formed by the four transmembrane
375 domains, as observed for the tetraspanin CD81^{34,63}. Our data suggest that this mechanism also
376 applies to CD63, contributing to cholesterol sorting into EVs. However, the sensitivity and
377 limitations of the various cholesterol probes used did not allowed the determination of the
378 precise mechanism by which CD63 manages cholesterol within the membrane. We cannot
379 discern whether CD63's role involves cholesterol translocation from the inner to the outer leaflet
380 of the membrane bilayer or cholesterol clustering in membrane microdomains, as both
381 processes are likely interdependent. We propose then that CD63 generates microdomains
382 enriched in cholesterol at endosome delimiting membranes that undergo inward budding to
383 generate cholesterol enriched ILVs. It remains uncertain whether a similar mechanism extends
384 to other tetraspanins reported to regulate cholesterol and oxysterols, such as CD81, CD82, and
385 TSPAN6^{34,35,63, 64}.

386 This clustering step may contribute to initial membrane bending to generate a subpopulation of
387 ILVs^{18,65} but may require additional machinery. The syntenin-alex pathway represents a
388 potential candidate, considering CD63's direct interaction with syntenin⁶⁶ and its promotion of
389 ESCRT-independent sorting¹⁸ at the expense of ESCRT-I-dependent sorting^{5,19,65} and the role
390 of syntenin in CD63 sorting to ILVs^{5,67}. As CD63 transiently traffics through the plasma
391 membrane, we cannot exclude that, there, it could act on subdomains that would bud as
392 ectosomes. Yet its primary action at MVEs is supported by its major localization in intracellular
393 compartments⁶⁸ and its effect on cholesterol levels predominantly in intracellular
394 compartments.

395 Our data strongly suggests that ILVs provide a temporary storage site for cholesterol that can
396 be retrieved by NPC1 pathway. The increased expression of CD63 in NPC1^{-/-} microglia would
397 suggest that both mechanisms are tightly intricate⁷⁰. The release of these ILVs as EVs further
398 support the role of exosomes in clearing excess cholesterol during pathological conditions⁶⁹.
399 While they may represent a subset of sEVs²⁸, we show here that they have the capacity to deliver
400 cholesterol to recipient cells via the NPC1/2 machinery of recipient lysosomes. This function
401 expands the ways in which EVs function as intercellular shuttles and underscores the emerging
402 role of EVs as trophic support⁶⁷. The importance of this alternative cholesterol source under
403 normal conditions is unclear. However, it could be crucial during starvation, aligning with
404 CD63's role in autophagy⁷¹ and likely influencing NPC1-dependent mTORC1 activity⁷² or in
405 the context of cancer, where part of the tumor might not be reached by the blood circulation.

406 In the absence of CD63, cholesterol is depleted from MVEs and accumulates in the Golgi.
407 While CD63 depletion does not affect the anterograde transport of VSVG⁷³, our data reveal that
408 cholesterol exits endosomes via tubular/vesicular carriers dependent on actin polymerization
409 for their fission. The mechanism behind endosomal tubulation remains unclear but may be
410 linked to impaired retromer function in NPC null cells⁷⁴. This retrograde transport pathway
411 could serve as a rescue mechanism to prevent cholesterol accumulation in endosomes,
412 highlighting the role of retrograde transport in endosomal cholesterol homeostasis⁷⁵.
413 Unexpectedly, we observed delayed retrograde trafficking of CI-M6PR in CD63 KO cells,
414 suggesting that the observed retrograde transport of cholesterol and associated proteins occurs
415 at the expense of the conventional pathway. Normal levels of free cholesterol are required for
416 CI-M6PR egress from endosomes⁴⁵, suggesting that altered cholesterol levels in the absence of
417 CD63 may affect actin bundling and impair CI-M6PR's ability to recruit the retromer
418 complex^{74,76}. These findings suggest that CD63 primarily acts at the limiting membrane of
419 MVEs, regulating cholesterol availability and lipid microdomain generation⁴³ to balance
420 inward and outward budding within the endosomal pathway².

421 Previous studies have suggested diverse, cell-type-specific functions of CD63^{17,18,20,22}, which
422 seem contradictory to its ubiquitous presence in MVEs and EV subpopulations across various
423 cell types. Our current findings on CD63's fundamental role in regulating cholesterol
424 homeostasis shed new light on these discrepancies, as several CD63-dependent cargo
425 trafficking processes are linked to cholesterol. For instance, MHC-II, whose exosomal secretion
426 is modulated by CD63⁷⁷, contains a cholesterol binding domain⁷⁸ while CD63-dependent
427 sorting of ApoE²⁰ likely relies on ApoE's ability to associate with cholesterol, as seen during
428 HDL formation⁷⁹. Notably, ApoE is not expressed in HeLa cells, ruling out its involvement in
429 CD63-dependent cholesterol trafficking. Additionally, CD63-dependent clustering of P-
430 selectin at the plasma membrane¹⁷ relies on cholesterol and lipid rafts, and other CD63 partners,
431 such as Synaptotagmin VII and LMP1, contain palmitate moieties^{81,82} that interact with
432 cholesterol, potentially stabilizing their localization in CD63 and cholesterol-enriched domains.
433 We also identified cargoes whose expression or trafficking are dependent on cholesterol
434 (MMP12⁸³, BASP1⁸⁴). It is therefore tempting to propose that the diverse roles of CD63 in
435 distinct cell types could be supported by a central role in the regulation of cholesterol trafficking
436 although other cargoes, such as CXCR4, may only rely on their pairing with CD63 to be
437 targeted to endosomes in a syntenin-dependent manner^{85,86}. Whether the CD63-cholesterol
438 affects only the sorting of a precise set of transmembrane proteins, or their clustering within

439 membrane microdomains or the binding of soluble protein to the surface of EVs, remains an
440 open question.

441 Further elucidation of the mechanisms discussed here may have implications for pathogenetic
442 mechanisms associated with CD63⁸⁷ or with the interrelated functions of the retrograde
443 pathway⁸⁸ and cholesterol in neurodegenerative diseases or with specific upregulation of CD63
444 in aging²⁵ and in cancer cells⁸⁹. From a therapeutic perspective, understanding the functional
445 relationship between CD63 and cholesterol could enhance the use of CD63 as an engineered
446 molecular tool to transform EVs into therapeutic shuttles⁹⁰.

447

448

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472

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477 M.C.P., S.S. and W.H.R. performed and analyzed atomic force microscopy. F.D. and D.L.

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479 lipidomic analysis and/or analyzed lipidomic data. F.V., S.C., M.T. and E.R. performed

480 experiments and/or generated tools/cell lines. G.R. and G.vN. acquired fundings. G.vN.

481 conceived and supervised the study. All authors read and approved the manuscript.

482

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487 **Competing interests**

488 The authors declare no competing interests.

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501 **Figure legends**

502

503 **Figure 1. Characterization of WT and CD63 KO HeLa and MNT-1 cells and sEVs.**

504 **a.** Western blot analysis of cell lysates derived from WT or CD63 KO HeLa or MNT-1 cells.
505 Quantification of protein content normalized to HeLa (n= 3 independent experiments, 2way
506 ANOVA, **** P<0.0001) or MNT-1 WT (n= 3 independent experiments, multiple unpaired t-
507 test, Holm-Šídák method, **** P< 0.0001). **b.** Western blot analysis of sEVs derived from WT
508 or CD63 KO HeLa or MNT-1 cells and isolated by UC. Quantification of protein content
509 normalized to HeLa (n= 3 independent experiments, 2way ANOVA, *** P= 0.0002 and ***P=
510 0.0003) or MNT-1 WT (n= 3 independent experiments, multiple unpaired t-test, Holm-Šídák
511 method, * P=0.006349, **** P<0.0001). **c.** NTA analysis of sEVs derived from WT or CD63
512 KO HeLa cells and isolated by UC. Average number of particles/ml (n= 3 independent
513 experiments, RM one-way ANOVA, ns P> 0.05) or size distribution (n= 3 independent
514 experiments, 2way ANOVA, only significant results are shown, * P= 0.0465, *** P= 0.0005).
515 **d.** NTA analysis of sEVs derived from MNT-1 cells WT or CD63 KO and isolated by SEC.
516 Average number of particles/ml (n= 6 independent experiments, two-tailed unpaired t test, ns
517 P= 0.9987) or size distribution (6 independent experiments, multiple unpaired Student's t test,
518 only significant results are shown). **e.** IFM of HeLa cells WT or CD63 KO stained for LAMP-
519 1. Bars=10 µm. Representative of 3 independent experiments. **f.** Electron micrograph of HeLa
520 cells WT or CD63 KO. Bars=200 nm. Representative of 2 independent experiments.
521 Data are presented as mean values +/- SEM. Source numerical data and unprocessed blots are
522 available in source data.

523

524 **Figure 2. Proteome analysis of WT and CD63 KO HeLa and MNT-1 cells and sEVs.**

525 **a.** Correlation plot of HeLa WT and MNT-1 WT cell proteomes expressed as fold changes of
526 peptide abundance (n= 5 independent experiments for cell lysates). **b.** List of proteins enriched
527 in WT cell proteomes and common between HeLa and MNT-1. **c.** Correlation plot of HeLa WT
528 and MNT-1 WT sEV proteomes expressed as the fold changes of peptide abundance (n= 5
529 independent experiments for sEVs). **d.** List of proteins enriched in WT sEV proteomes and
530 common between HeLa and MNT-1. List of Molecular function GO Terms common between
531 HeLa WT and MNT-1 WT sEVs.
532 A linear model (adjusted on peptides and biological replicates) was performed, and a two sided
533 t-test was applied on the fold change estimated by the model. The p-values were then adjusted

534 using the Benjamini–Hochberg FDR procedure. Source numerical data are available in source
535 data.

536

537 **Figure 3. Lipidome analysis of WT and CD63 KO HeLa and MNT-1 cells and sEVs.**

538 **a.** Comparison of lipid class levels (expressed as mol% of total lipids) identified in WT HeLa
539 cells or sEVs (n= 3 independent experiments for cell lysates and 4 independent experiments for
540 sEVs, two-tailed Student's t test). **b.** Comparison of lipid class levels (mol% of total lipids)
541 identified in WT MNT-1 cells or sEVs (n= 3 independent experiments for cell lysates and 5
542 independent experiments for sEVs, two-tailed Student's t test). **c.** Comparison of lipid class
543 levels (mol% of total lipids) identified in WT or CD63 KO HeLa cells (n= 3 independent
544 experiments for WT cells and 7 independent experiments for KO cells, two-tailed Student's t
545 test). **d.** Comparison of lipid class levels (mol% of total lipids) identified in WT or CD63 KO
546 MNT-1 cells (n= 3 independent experiments for WT cells and 3 independent experiments for
547 KO cells, two-tailed Student's t test). **e.** Comparison of lipid class levels (mol% of total lipids)
548 identified in sEVs derived from WT or CD63 KO HeLa cells (n= 4 independent experiments
549 for WT sEVs and 8 independent experiments for KO sEVs, two-tailed Student's t test). **f.**
550 Comparison of lipid class levels (mol% of total lipids) identified in sEVs derived from MNT-1
551 cells WT or CD63 KO (n= 5 independent experiments for WT sEVs and 5 independent
552 experiments for KO sEVs two-tailed Student's t test). *P* value: **P* < 0.05, ***P* < 0.01, ****P*
553 < 0.001. Data are presented as mean values +/- SEM. Source numerical data are available in
554 source data.

555 List of lipids: Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol
556 (PI), Plasmalogen PE (PE-P), Phosphatidylserine (PS), Phosphatidylglycerol (PG),
557 Phosphatidic acid (PA), Bis(monoacylglycero)phosphate (BMP), Lysophosphatidylcholine
558 (LPC), Lysophosphatidylethanolamine (LPE), Lysophosphatidylglycerol (LPG),
559 Sphingomyelin (SM), Ceramide (Cer), Dihydroceramide (DHC), Cholesterol (FC), Cholesteryl
560 esters (CE).

561

562 **Figure 4. Cholesterol localization in WT and CD63 KO HeLa cells.**

563 **a.** Localization of LDL-BODIPY in WT and CD63 KO HeLa cells treated with vehicle or
564 U18666A. Bars=10 μm. Quantification of LDL-BODIPY fluorescence intensity (n= 15 cells
565 pooled across 1 independent experiment, Kruskal-Wallis test, *** *P* = 0.0006, **** *P* < 0.0001).
566 **b.** Localization of cholesterol stained with filipin in WT or CD63 KO HeLa cells treated with
567 vehicle or U18666A. Bars=10 μm. Quantification of filipin fluorescence intensity in HeLa cells

568 treated with U18666A, expressed as relative enrichment compared to vehicle-treated cells (n=
569 18 cells pooled across 2 independent experiments, Kruskal-Wallis test, * P= 0.0281). **c.**
570 Localization of cholesterol in HeLa cells WT or CD63KO treated with vehicle or U18666A,
571 permeabilized and stained with D4-GFP. Bars=10 μ m. Quantification of D4-GFP fluorescence
572 intensity expressed as integrated density of fluorescence within cells (n=9 WT cells, 7 KO cells,
573 21 WT + U18666A cells, 28 KO + U18666A cells pooled across 3 independent experiments,
574 Ordinary one-way ANOVA, ns P> 0.005, * P= 0.0148 or 0.0101, ** P= 0.0048). **d.**
575 Colocalization of cholesterol and CD63 positive compartments in HeLa cells permeabilized and
576 stained with D4-GFP and CD63. Representative of 3 independent experiments. **e.** Localization
577 of cholesterol in WT or CD63 KO HeLa cells transfected with pD4-mCherry, treated with
578 vehicle or U18666A and stained for CD63. Bars=10 μ m. Quantification of D4-mCherry
579 fluorescence intensity in HeLa cells treated with vehicle (n=30 cells pooled across 3 independent
580 experiments, Mann-Whitney test, ** P= 0.0019) or U18666A (n=30 cells pooled across 3
581 independent experiments, t-test, **** P <0.0001). **f.** HeLa cells WT or CD63 KO were grown
582 in LPDS, processed for ultrathin cryo-sectioning and immuno-gold labeled with D4-GFP (PAG
583 10nm) and CD63 (PAG 5nm). Bars=200 nm. MVEs containing cholesterol are shown. Bars=
584 200 nm. Representative of 3 independent experiments, quantified in **(h)**. **g.** HeLa cells WT or
585 CD63 KO were grown in LPDS, processed for ultrathin cryo-sectioning and immuno-gold
586 labeled with D4-GFP (PAG 10nm). White arrows indicate MVEs containing cholesterol, black
587 arrows indicate Golgi apparatus, and arrowheads indicate transport vesicles containing
588 cholesterol. Bars=200 nm. Representative of 3 independent experiments, quantified in **(h)**. **h.**
589 Quantification of the number of gold particles on intraluminal vesicles (ILVs) per MVE, MVE
590 limiting membrane (LM) and Golgi apparatus (MVEs, H WT n=92, H CD63 KO#1 n=41, H
591 CD63 KO#2 n=115; Golgi, H WT n=18 H CD63 KO#1 n=17, H CD63 KO#2 n=34; pooled
592 across 3 independent experiments, 2-way ANOVA multiple comparisons, ns P > 0.05, ****
593 P< 0.0001). Data are presented as mean values +/- SEM. Source numerical data are available
594 in source data.

595

596 **Figure 5. Absence of CD63 induces accumulation of cholesterol at the Golgi apparatus**
597 **and actin-dependent endosomal tubulations.**

598 **a.** Localization of TF-chol in WT and CD63 KO HeLa cells treated with vehicle or U18666A.
599 Bars=10 μ m. Red circle indicates perinuclear accumulation of TF-chol. A 3D view of HeLa
600 cells treated with U18666A is shown in the bottom panel, with white arrows indicating the
601 perinuclear accumulation of TF-chol. Representative of 3 independent experiments quantified

602 in **(b,c)** **b.** Quantification of TF-chol fluorescence intensity in positive compartments of WT or
603 CD63 KO HeLa cells treated with vehicle or U18666A (n= 35 cells pooled across 3 independent
604 experiments, Kruskal-Wallis test, ns $P > 0.9999$, ** $P = 0.0025$, *** $P = 0.0001$, **** $P < 0.0001$).
605 Quantification of the size (n=35 cells pooled across 3 independent experiments, Kruskal-Wallis
606 test, ns $P = 0.6459$, * $P = 0.0276$, ** $P = 0.0050$, **** $P < 0.0001$) and the number of TF-chol
607 positive compartments in WT or CD63 KO HeLa cells treated with vehicle or U18666A. (n=35
608 cells pooled across 3 independent experiments, Kruskal-Wallis test, ns > 0.005 , ** $P = 0.0041$,
609 **** $P < 0.0001$). **c.** Quantification of the localization of TF-chol positive compartments within
610 WT or CD63 KO HeLa cells treated with vehicle or U18666A. Data are expressed as percentage
611 of compartments in the perinuclear area or in the periphery (n=25 cells pooled across 3
612 independent experiments, 2-way ANOVA, ns $P > 0.005$, * $P = 0.0260$, ** $P = 0.0046$, *** $P =$
613 0.0005). **d-e.** Localization of TF-chol in WT and CD63 KO HeLa cells transfected with the
614 Golgi marker Mann-II-mCherry (**d**) or with the late endosomal marker LAMP-1-mCherry (**e**)
615 and treated with U18666A. Bars=10 μm . Manders' colocalization coefficient between TF-chol
616 and Mann-II 1 (n=13 WT cells, n= 12 KO cells pooled across 2 independent experiments, two-
617 tailed unpaired t-test, *** $P = 0.0009$) or LAMP-1 (n= 17 WT cells, 15 KO cells pooled across
618 2 independent experiments, two-tailed unpaired t-test, * $P = 0.0104$). **f.** Electron micrograph of
619 HeLa cells WT or CD63 KO analyzed by 2D EM. Bars= 200 nm. Quantification of the number
620 of tubules/buds per endosome (n= number of MVEs, H WT n=24, H CD63 KO#1 n=22, H
621 CD63 KO#2 n=18; pooled across 2 independent experiments, Ordinary one-way ANOVA, ns
622 $P = 0.9033$, ** $P = 0.0062$ and 0.0067). **g.** SR-IFM of HeLa cells WT or CD63 KO treated with
623 DMSO or CK666 and co-stained for LAMP-1 and actin. Bars= 10 μm , bars= 5 μm for
624 magnifications. Normalized phalloidin fluorescence on LAMP-1-positive endosomes in HeLa
625 cells treated with DMSO or CK666 (n=number of endosomes, H WT DMSO n=816, H CD63
626 KO#1 DMSO n=725, H CD63 KO#2 DMSO n=974, H WT CK666 n=341, H CD63 KO#1
627 CK666 n=385, H CD63 KO#2 CK666 n=258; pooled across 3 independent experiments,
628 Kruskal-Wallis test, *** $P = 0.0006$, **** $P < 0.0001$). Data are presented as mean values +/-
629 SEM. Source numerical data are available in source data.

630

631 **Figure 6. CD63 presents an intramembrane cavity able to accommodate cholesterol.**

632 **a.** Modeling of CD63 WT structure generated with I-TASSER suite (C-score= 0.23) (rainbow
633 colors), superimposed on CD81 structure (purple sharp line). **b.** Sequence alignment of CD63,
634 CD81, and CD9. Sequences were obtained from Uniprot and alignment was performed using
635 CLUSTAL O (1.2.4). Red square represents the glutamate residue in CD63 (E217) that was

636 mutated in this study. Green square represents the glutamate residue in CD81 (E219) that was
637 mutated in CD81³⁴. Blue squares indicate the regions corresponding to the transmembrane
638 domains. **c.** Modeling of CD63 WT or E217Q interaction with a cholesterol molecule created
639 with Haddock 2.4 suite. Magnification shows CD63 residues predicted to interact with
640 cholesterol (yellow structure). **d.** Western blot analysis of cell lysates and sEVs from WT or
641 CD63 KO or CD63 KO HeLa cells rescued with CD63 WT or mutant E217Q. Representative
642 of 3 independent experiments. **e.** CD63 KO HeLa cells were grown in LPDS, rescued with
643 CD63 WT or mutant E217Q, processed for ultrathin cryo-sectioning and immuno-gold labeled
644 with D4-GFP (PAG 10nm) and CD63 (PAG 5nm). Bars=200 nm. Representative of 2
645 independent experiments. **f.** Localization of cholesterol stained with filipin in HeLa cells CD63
646 KO rescued with CD63 WT or CD63 E217Q and treated with vehicle or U18666A. Bars=10
647 μ m. Quantification of filipin fluorescence intensity in HeLa cells treated with U18666A (n=18
648 cells pooled across 2 independent experiments, Kruskal-Wallis test, ns $P > 0.9999$). **g.**
649 Localization of CD63 and recombinant D4-GFP in HeLa CD63 KO cells rescued with CD63
650 WT or CD63 E217Q and treated with U18666A. Bars=10 μ m. Quantification of D4-GFP
651 integrated density of fluorescence (n= 13 CD63 KO + CD63 WT cells, 9 CD63 KO + CD63
652 E217Q cells pooled across 2 independent experiments, two-tailed Mann-Whitney test, ** $P =$
653 0.0071). **h.** Localization of CD63 and expressed D4-mCherry in HeLa cells CD63 KO cells
654 rescued with CD63 WT or CD63 E217Q and treated with U18666A. Bars =10 μ m.
655 Quantification of D4-mCherry integrated density of fluorescence (n= 17 CD63 KO + CD63
656 WT cells, 21 CD63 KO + CD63 E217Q cells pooled across 3 independent experiments, two-
657 tailed t-test, ** $P = 0.0072$). Data are presented as mean values \pm SEM. Source numerical data
658 and unprocessed blots are available in source data.

659 **Figure 7. CD63 modulates cholesterol trafficking to sEVs and exosomes, affecting their**
660 **biophysical properties.**

661 **a.** sEVs were isolated from HeLa cells (grown in LPDS) WT or CD63 KO or CD63 KO rescued
662 with CD63 WT or CD63 E217Q and observed by electron microscopy after immunogold
663 labeling with D4-GFP (PAG 10nm). Bars=200 nm. **b.** Quantification of the number of gold
664 particles per EV (n=number of sEVs, H WT n=1201, H CD63 KO#2 n=942, pooled across 4
665 independent experiments, H CD63 KO#1 n=524, H CD63 KO#2+CD63 WT n=1368, H CD63
666 KO#2+CD63 E217Q n=1035 pooled across 2 independent experiments, Kruskal-Wallis test, ns
667 $P > 0.9999$, ns $P = 0.1275$, * $P = 0.0100$, **** $P < 0.0001$). **c.** sEVs were isolated from MNT-1
668 cells WT or CD63 KO grown in LPDS and observed by electron microscopy after immunogold

669 labeling with D4-GFP (PAG 10nm). Bars=200 nm. Quantification of the number of gold
670 particles per EV (n=number of sEVs, M WT n=509, M CD63 KO n=426; pooled across 2
671 independent experiments, two-tailed Mann-Whitney test, **** P< 0.0001). **d.** Cholesterol
672 content of sEVs isolated from Bafilomycin-A1 treated HeLa cells (grown in LPDS) WT or
673 CD63 KO and measured by Amplex Red assay (including or not cholesterol esterase).
674 Cholesterol content is normalized to the number of vesicles (g of chol per single sEV). (n= 4
675 for WT, n=5 for KO independent experiments for condition without esterase, two-tailed Mann
676 Whitney test, * P=0.0159; n= 7 independent experiments for condition with esterase, two-tailed
677 unpaired t test, * P= 0.0104). **e.** AFM topography image of H WT sEVs (left) and H CD63
678 KO#2 sEVs (right) attached to glass coverslip coated with poly-L-lysine (PLL). Color scale
679 indicates height. **f.** Deformation distribution of sEVs upon substrate adhesion determined by
680 their height (H) over radius of curvature (R_c): The averaged ratio was 1.41 ± 0.06 (S.E.M.,
681 N=42 sEVs) for H WT and 1.35 ± 0.04 (S.E.M., N=45 sEVs) for H CD63 KO#2. The inset in
682 the lower left corner illustrates reference shapes for H/ R_c equals 0.5, 1, and 2. Boxplots are
683 shown in which the center line indicates median, box limits indicate upper and lower quartiles
684 and whiskers indicate 1.5x interquartile range. **g.** Distribution of calculated radii R_0 of the sEVs
685 while in solution. Mean radius of the sEVs is 64 ± 4 nm (S.E.M., N=42 sEVs) for H WT sEVs
686 and 69 ± 2 nm (S.E.M., N=45 sEVs) for H CD63 KO#2 sEVs. **h.** Stiffness of sEVs obtained by
687 linearly fitting force indentation curves between $0.02 - 0.1 R_c$. Mean values are 0.010 ± 0.002
688 N/m (S.E.M., N=42 sEVs) for H WT sEVs, and 0.010 ± 0.001 N/m (S.E.M., N=45 sEVs) for
689 H CD63 KO#2 sEVs. Boxplots are shown in which the center line indicates median, box limits
690 indicate upper and lower quartiles and whiskers indicate 1.5x interquartile range. **i.**
691 Dimensionless pressure vs. dimensionless stiffness for sEVs. The theoretically predicted curve
692 is displayed as solid red line. Data for H WT sEVs and H CD63 KO#2 sEVs were individually
693 fitted to the theoretical curve with the bending modulus κ as parameter. Bending moduli
694 obtained for H WT sEVs and H CD63 KO#2 are $\kappa = (11 \pm 3) k_B T$ (S.E.M, N = 36 sEVs), and
695 $\kappa = (14 \pm 4) k_B T$ (S.E.M., N = 30 sEVs), respectively. The error was determined using the bias
696 corrected percentile method with 500 bootstrapping repetitions. A two-sided t-test revealed a
697 significant difference between the bending moduli (p = 0.05).
698 Data are presented as mean values +/- SEM. Source numerical data are available in source data.
699

700 **Figure 8 . CD63 sEVs can transfer cholesterol to recipient cells.**

701 **a.** WT or CD63 KO HeLa sEVs were labeled with MemBright-640 and incubated overnight
702 with WT or CD63 KO HeLa cells. Bars= 10 μ m. Quantification of MemBright-640 intensity in

703 WT and CD63 KO HeLa cells (n=10 cells pooled across 2 independent experiments, Ordinary
704 One-way ANOVA test, ns $P > 0.05$, * $P=0.0328$). **b.** WT HeLa cells were labeled with TF-chol,
705 sEVs containing TF-chol were isolated, labeled with MemBright-640, and incubated overnight
706 with WT or CD63 KO HeLa cells treated with vehicle or U18666A. Bars= 10um.
707 Quantification of Membright-640 intensity in recipient WT and CD63 KO HeLa cells treated
708 or not with U18666A (n=10 cells pooled across 2 independent experiments, Kruskal-Wallis
709 test, ns $P > 0.9999$, ns $P = 0.3203$, ns $P= 0.5110$, ns $P= 0.7278$). Quantification of TF- chol
710 intensity in recipient WT and CD63 KO HeLa cells treated or not with U18666A (n=12 cells
711 pooled across 2 independent experiments, Kruskal-Wallis test, ns $P=0.6785$, ns $P>0.9999$, *
712 $P=0.05$, ** $P=0.0028$, **** $P <0.0001$). **c.** WT or CD63 KO HeLa cells were labeled with TF-
713 chol, sEVs containing TF-chol were isolated, and incubated overnight with CD63 KO HeLa
714 cells treated with vehicle or U18666A. Bars=10 μ m. Quantification of TF-chol intensity in
715 recipient WT or CD63 KO HeLa cells. (n=9 cells pooled across 2 independent experiments,
716 Ordinary One-way ANOVA test, * $P= 0.0431$, ** $P= 0.0097$, *** $P= 0.0006$, **** $P < 0.0001$).
717 Data are presented as mean values +/- SEM in **a** and **b**. Data in **c** are presented as violin plot
718 with median and quartiles. Source numerical data are available in source data.
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721 **References**

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925 sensitive bioluminescence imaging. *J. Extracell. Vesicles* **9**, 1800222 (2020).
- 926
- 927

928 **Methods**

929

930 **Cell culture, transfection, and drug treatments**

931 HeLa Kyoto were originally obtained from ATCC and gifted to us by Dr. Bruno Goud's
932 laboratory (CNRS UMR144, Institut Curie, Paris, France). MNT-1 cells were originally
933 obtained from ATCC and gifted to us by Dr. Michael S. Marks (Children's Hospital of
934 Philadelphia, Philadelphia, USA). HeLa cells were cultured in DMEM Glutamax (Gibco)
935 supplemented with 10% (v/v) FBS, 1% penicillin-streptomycin (Gibco). MNT-1 cells were
936 cultured in DMEM Glutamax (Gibco) supplemented with 20% FBS, 10% AIM-V medium,
937 sodium pyruvate, nonessential amino acids, and penicillin-streptomycin (Gibco). Where
938 indicated, FBS was substituted by LPDS 1/30 (Millipore Merck). All cells were maintained at
939 37°C in a 5% (v/v) CO₂ incubator. For siRNA transfection, MNT-1 cells were seeded at day 1,
940 transfected with siRNAs (200 pmol) at day 3 and at day 5 using Oligofectamine (Thermo Fisher
941 Scientific), according to the manufacturer's instructions, and analyzed at day 7. For rescue
942 experiments, HeLa cells were microporated using the Neon Electroporation Transfection
943 System (Invitrogen, Thermo Fisher Scientific) with 0.5 ug of plasmid according to the
944 manufacturer's protocol using the following settings: two pulses at 1.005 V with 35-ms pulse
945 width. For plasmid transfection, cells were transfected using JetPrime Transfection reagent
946 (Polyplus-Transfection) according to the manufacturer's protocol.
947 CK666 (Tocris Biosciences) treatments were for 2 h at 200 μM. U18666A (Sigma Aldrich
948 chimie) treatments were for 16h at 1 μM. Bafilomycin-A1 (Sigma Aldrich chimie) treatments
949 were for 2 h at 100 nM.

950

951 **Generation of CRISPR/CAS9 CD63 KO**

952 CD63 KO HeLa or MNT-1 cells were generated using CRISPR/Cas9 gene editing technology.
953 The target sequence (CCAGTGGTCATCATCGCAGT) was selected using the CRISPR design
954 tool available at the Broad Institute ([https://portals.broadinstitute.org/gpp/public/analysis-](https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design)
955 [tools/sgrna-design](https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design)). The corresponding guide DNA sequences were cloned into the
956 lentiCRISPRv2 plasmid (Addgene#52961) according to the instructions of the Zhang
957 laboratory (<http://www.genome-engineering.org/gecko/>)⁹¹. The plasmid was transfected using
958 FuGENE HD according to the manufacturer's instructions for HeLa cells, and the Neon
959 Electroporation Transfection System (Invitrogen, Thermo Fisher Scientific) for MNT-1 cells.
960 MNT-1 cells were microporated with 0.5 ug of plasmid following optimized settings: three
961 pulses at 1.400 V with 10-ms pulse width. After 36-48h, cells were treated with 10 μg/ml

962 puromycin for 48h. Cells negative for CD63 were sorted following staining with the CD63
963 mAb TS63 using a FACS Aria cell sorter (Beckton Dickinson). CD63 KO HeLa clones #1 and
964 #2 were obtained by single-cell culture in 96-well plates.

965

966 **Reagents, antibodies, plasmids, and siRNAs**

967 Reagents were obtained from the following sources: recombinant D4-GFP was a kind gift of
968 Fabien Alpy (IGBMC, Illkirch, France), phalloidin conjugated to Alexa Fluor 647, LDL-
969 Bodipy, and Nile Red from Thermo Fisher Scientific, (810255P) and Filipin III (F4767) from
970 Sigma Aldrich chimie.

971 Antibodies were obtained from the following sources: anti-CD63 (CLB-180, Thermo Fisher
972 Scientific, or ab23792, Abcam, 1:200 for WB, IF and EM), anti-CD9 (mAb TS9) and anti-
973 CD63 (mAb TS63) were a kind gift of Eric Rubinstein (CIMI, Paris, France), anti-alex
974 (pab0204, Covalab, 1:500 for WB), anti- syntenin (ab19903, Abcam, 1:500 for WB), anti-
975 tubulin (ab6046, Abcam, 1:10,000 for WB), anti- β -actin (A5316, Sigma, 1:2000 for WB), anti-
976 ApoE (ab52607, Abcam, 1:500 for IF and WB), anti-EEA1 (ab70521, Abcam, 1:200 for IF),
977 anti-TGN46 (AHP500GT, Biorad, 1:300 for IF), anti-GM130 (610822, BD Transduction
978 Laboratories, 1:1000 for IF), anti-CI-M6PR (ab8093, Abcam, 1:100 for pulse/chase), anti-
979 LAMP-1 (555798, BD Biosciences, 1:200 for IF), anti-GFP (A11122, Thermo Fisher
980 Scientific, 1:400 for EM), anti-SREBP2 (Clone # 751512, MAB7119, R&D Systems, 1:250 for
981 WB).

982 Secondary anti-rabbit IgG or anti- mouse IgG anti-sheep IgG antibodies conjugated to Alexa
983 Fluor were used 1:200 (AF488: anti-mouse A-11029, anti-rabbit A11008; AF555: anti-rabbit
984 A-21429; AF647: anti-rabbit A21245, anti-mouse A-21236,) from Thermo Fisher Scientific,
985 anti-sheep AF647 (ab150179) from Abcam. HRP-conjugated goat anti-mouse (A-5278), anti-
986 rabbit (A-6154) were from Sigma and used 1:10,000. Protein A conjugated to 5 or 10 nm gold
987 particles (PAG; used 1:50; Cell Microscopy Center, Utrecht University Hospital, Utrecht,
988 Netherlands).

989 The sense strand for the indicated double-stranded siRNAs were synthesized with the following
990 sequences (Qiagen): siCtrl, 5'-AATTCT CCG AAC GTG TCA CGT-3'; siCD63, 5'-
991 AAGTTCTTGCTCTACGTCCTC-3'.

992 CD63-E217Q was constructed by Gibson Assembly using the primer pairs CD63E217Q_fwd
993 5'-CTTGAATTGCTTTTGTCCAGGTTTTGGGAATTGTCTTTG-3' and CD63E217Q_rev
994 5'- CAAAGACAATTCCCAAACCTgGACAAAAGCAATTCCAAG-3' and AmpR_fwd
995 5'-CTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTC-3' and

996 AmpR_rev 5'-GACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAG-3'
997 on the parental vector pCDNA3.1-CD63 (CD63-WT) (kind gift from Kei Sato, University of
998 Tokyo).
999 Mann-II-mCherry-pCMV, Lamp1-mCherry-pCMV and VSVG-EGFP-RUSH were kindly
1000 provided by Gaëlle Boncompain and Franck Perez (Institut Curie, Paris, France), D4-mCherry
1001 was kindly provided by Fabien Alpy (IGBMC, Illkirch, France)⁹².

1002

1003 **sEV isolation and characterization**

1004 sEVs were prepared from conditioned media incubated for 48h on sub-confluent cells. FBS
1005 supplemented medium was previously centrifuged at 100,000 g for 16h to remove FBS-derived
1006 EVs. Conditioned media were centrifuged at 300 g (15 min, 4° C) and 2000 g (20 min, 4°C) to
1007 remove cell debris.

1008 For isolation by differential ultracentrifugation (UC), the supernatant (post 2000 g) was
1009 centrifuged at 10,000 g (30 min, 4°C) and sEVs were collected from the supernatant by
1010 centrifugation at 100,000 g (60 min, 4°C, 45Ti or 70Ti rotor, Beckman Coulter). The pellet was
1011 washed in PBS (pH 7) by centrifugation at 100,000 g (60 min, 4°C) and finally resuspended in
1012 PBS (pH 7).

1013 For isolation by size exclusion chromatography (SEC), the supernatant (post 2000 g) was
1014 concentrated by ultrafiltration on a 10,000 MWCO filter (Centricon, Merck-Millipore) to obtain
1015 a concentrated conditioned media (CCM). sEVs were then isolated by SEC (IZON Science).
1016 Fractions from 7 to 9 were collected, pooled, and concentrated again using a 10,000 MWCO
1017 filter (Centricon, Merck-Millipore).

1018 EV samples were used right after isolation or stored at -80°C.

1019 For D4-GFP staining of sEVs, CCM (obtained as above) was incubated with D4-GFP (dilution
1020 1:1000) overnight at 4°C under rotation and then fixed with 2% PFA for 15 minutes. sEVs were
1021 then isolated by SEC as described above. The samples were then processed for electron
1022 microscopy as described below.

1023

1024 **Nanoparticle-tracking analysis**

1025 NTA measurements of EV size and concentration were performed using a ZetaView®, Particle
1026 Metrix. Samples were pre-diluted with filtered PBS and measured at 11 different positions for
1027 2 cycles at 24°C. The videos were then processed by the ZetaView Software (version 8.04.02
1028 SP).

1029

1030 **Dynamic Light Scattering**

1031 DLS measurement of EV concentration was performed using Zetasizer Ultra®, Malvern
1032 Panalytical and ZS XPLOERER (version 2.0.0.98) acquisition software. Samples were loaded in
1033 low-volume quartz batch ZEN 2112 cuvette (Hellma® Analytix, QS High Precision Cell, light
1034 path 3x3 mm) for analysis at room temperature with a dispersant scattering value of 82.
1035 Distributed particle concentration was measured.

1036

1037 **Western Blot**

1038 Cells were lysed on ice in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, and 1
1039 mM EDTA, pH 7.2) supplemented with a protease inhibitor cocktail (Roche). Cell lysates or
1040 EVs were incubated with sample buffer with or without 350 mM 2-mercaptoethanol (Sigma),
1041 incubated at 60°C for 30 min, loaded on 4–12% Bis-Tris gels (Nu-PAGE, Invitrogen), and
1042 transferred on nitrocellulose membranes (GE Healthcare). Membranes were blocked in
1043 PBS/0.1% Tween (PBS/T) with 5% nonfat dried milk, incubated with indicated primary and
1044 secondary antibodies diluted in PBS/T-milk. Western blots were developed using the ECL
1045 SuperSignal West Pico or Dura (Thermo Fisher Scientific). Signal intensities were quantified
1046 with Image J Fiji software (version 2.1.0/1.53c or 1.54f).

1047

1048 **Immunofluorescence and microscopy**

1049 Cells were grown on coverslips and fixed for 20 minutes at RT with 4% PFA/PBS and quenched
1050 for 10 min with PBS/50 mM glycine. Cells were incubated with PBS/0.2% BSA/0.1% saponin
1051 before incubation with primary and secondary antibodies in the same buffer. For ApoE staining,
1052 cells were fixed with cold methanol for 5 min at -20°C and IF was performed using PBS/0.2%
1053 BSA. Incubation with phalloidin AF-647 was performed during the secondary antibody
1054 incubation.

1055 For CI-M6PR pulse-chase, cells grown on coverslips were incubated for 15 minutes at RT with
1056 anti-CI-M6PR antibody in complete media, washed with PBS and incubated at 37°C for 1h to
1057 allow antibody internalization. Cells were fixed as described above and processed for IF with a
1058 secondary antibody anti-mouse.

1059 For VSVG-RUSH experiment, cells grown on coverslips were transfected with VSVG-EGFP-
1060 RUSH. After 20 h, D-biotin (40 µM, T=0) was added, cells were fixed at T=15, 30 or 60
1061 minutes.

1062 For Nile Red staining, cells were grown on coverslips and fixed as above. Cells were incubated
1063 with Nile Red for 5 minutes.

1064 Coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen).
1065 Coverslips were analyzed on a wide-field microscope (Eclipse 80i Upright; Nikon) equipped
1066 with a CCD camera (CoolSNAP HQ2; Photometrics) using a 100x 1.4 NA Plan-Apo objective
1067 lens. Z images series were acquired every 0.2 μm with the piezoelectric motor (Physik
1068 Instrumente) using MetaMorph software (Molecular Devices, version 7.8.0.0). Raw images
1069 were deconvolved with Meinel algorithm (PICT-IBiSA imaging platform at Institut Curie).
1070 For super-resolution microscopy (SR-IFM), cells were analyzed as previously described⁹³.
1071 Briefly, images were acquired on a spinning disk system (Gataca Systems) based on an inverted
1072 microscope (Ti-E; Nikon) equipped with a sCMOS camera (Prime 95B; Photometrics), a
1073 confocal spinning head (X1; Yokogawa), a 100x 1.4 NA Plan-Apo objective lens, and a
1074 superresolution module (Live-SR; Gataca systems) based on structured illumination with
1075 optical reassignment technique and online processing leading to a two-time resolution
1076 improvement. MetaMorph software (Molecular Devices, version 7.8.0.0) was used.
1077 For TopFluor Cholesterol (TF-chol) staining, cells were grown on coverslips in LPDS medium
1078 and incubated with TF-chol at 1 μM for 16h.
1079 For LDL-BODIPY endocytosis, cells were grown on coverslips in LPDS medium and
1080 incubated with 4 $\mu\text{g}/\text{mL}$ LDL-BODIPY for 16h before fixation.
1081 For filipin staining, cells were grown on coverslips in LPDS medium and treated or not with
1082 U18666A at 1 μM for 16h before fixation. Coverslips were then incubated with Filipin III at
1083 50 $\mu\text{g}/\text{mL}$ for 1h at RT in the dark before mounting. Coverslips were observed on a Leica 3D
1084 widefield 3D DEC microscope with a 100x 1.4 NA HCX Plan-Apo objective lens using a
1085 Metamorph (Molecular Devices) software.
1086 For D4-GFP staining, cells were grown on coverslips in LPDS medium and treated or not with
1087 U18666A at 1 μM for 16h followed by D4-GFP staining as previously described⁹². Briefly, for
1088 non-permeabilized cells, coverslips were incubated with D4-GFP for 30 minutes at 37°C before
1089 fixation and mounting. For permeabilized cells, cells were fixed, and permeabilized in liquid
1090 nitrogen before D4-GFP incubation, CD63 antibody staining and mounting. Images were
1091 acquired using a confocal microscope Leica SP5 equipped with a 63x 1.4 NA objective using
1092 a LAS-AF software (Leica version 2.7.3.9723).

1093

1094 **Electron Microscopy**

1095 For conventional electron microscopy, cells were seeded on coverslips and fixed with 2.5 %
1096 glutaraldehyde in 0.1 M cacodylate buffer. Cells were processed for Epon (TAAB Laboratories

1097 Equipment) embedding as described⁹⁴ and ultrathin sections were contrasted with uranyl acetate
1098 and lead citrate.

1099 For high-pressure freezing, cells were cultured on carbonated sapphire disks, high-pressure
1100 frozen with HPM100 (Leica Microsystems) and then freeze substituted in anhydrous acetone
1101 containing 1% OsO₄/2% H₂O for 64h in a freeze substitution system (AFS; Leica
1102 Microsystems). Cells were embedded in Epon and processed for sectioning and contrasting
1103 with uranyl acetate and lead citrate.

1104 For ultrathin cryosections and immunogold labeling, cells were grown on flasks and fixed with
1105 2% PFA/ 0.125% glutaraldehyde/ 0.1 M phosphate buffer. Cell pellets were embedded in 10%
1106 gelatin and infused in 2.3M sucrose. Gelatin blocks were frozen and processed for
1107 ultracryomicrotomy. Ultrathin sections were immunogold labeled using PAG 5 or 10 nm as
1108 previously described⁸². For cholesterol labeling, ultrathin sections were directly incubated for
1109 30 min with D4-GFP (dilution 1:500), fixed with 1% glutaraldehyde and immunogold labeled
1110 using an antibody anti-GFP and PAG 10 nm.

1111 EVs were directly deposited on formvar/carbon coated copper/palladium grids for 20 min at
1112 RT, fixed with PFA 2%/0.1 M phosphate buffer and processed for immunogold labeling as for
1113 cells.

1114 Negative staining of cell ultrathin cryo-sections or EVs was performed using uranyl acetate
1115 0.4% in methylcellulose.

1116 The samples were analyzed with an 80 kV transmission electron microscope (Tecnai Spirit G2;
1117 Thermo Fischer, Eindhoven, The Netherlands) equipped with a 4k CCD camera (Quemesa,
1118 EMSIS, Münster, Germany) using the software iTEM (v.5.2; EMSIS).

1119

1120 **Proteomic analysis**

1121 Sample preparation: HeLa or MNT-1 cells were cultured in media supplemented with LPDS
1122 for 48 hours, and lysed in freshly prepared urea buffer (8 M urea, 200mM ammonium
1123 bicarbonate). sEVs were isolated by SEC, fractions 7 to 9 were collected, pooled, centrifuged
1124 at 100,000g for 1h at 4°C, and the pellets were lysed in freshly prepared urea buffer. After
1125 sonication, lysates were quantified by *BCA* (ThermoScientific) or Nanodrop at 280nm
1126 wavelength (Mettler Toledo).

1127 After vacuum concentration to dryness, sEV samples were diluted in 20µl of 8 M urea, 200mM
1128 ammonium bicarbonate. Cell lysates and sEV samples were then reduced with 5 mM DTT for
1129 1 h at 37 °C and alkylated with 10 mM iodoacetamide for 30 min at room temperature in the
1130 dark. Samples were then diluted in 200mM ammonium bicarbonate to reach a final

1131 concentration of 1 M urea and digested overnight at 37 °C with Trypsin/Lys-C (Promega CAT#: V5071) at a ratio of 1/50 for proteome samples and with 0.2 µg of Trypsin/LysC for sEVs. 5µg of proteomes or EVs were then loaded onto a homemade C18 StageTips for desalting. Peptides were eluted using 40/60 MeCN/H₂O + 0.1% formic acid and vacuum concentrated to dryness. Peptides were reconstituted in 10 µl of injection buffer in 0.3% trifluoroacetic acid (TFA) before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

1137 LC-MS/MS Analysis: Online chromatography was performed with an RSLC nano system (Ultimate 3000, Thermo Scientific) coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific). Peptides were trapped on a C18 column (75 µm inner diameter × 2 cm; nanoViper Acclaim PepMapTM 100, Thermo Scientific) with buffer A (2/98 MeCN/H₂O in 0.1% formic acid) at a flow rate of 3.0 µL/min over 4 min. Separation was performed on a 50 cm x 75 µm C18 column (nanoViper Acclaim PepMapTM RSLC, 2 µm, 100Å, Thermo Scientific) regulated to a temperature of 40°C with a linear gradient of 3% to 29% buffer B (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 91 min for the EV analyses and a linear gradient of 3% to 32% buffer B over 211min for the cell proteome analyses. MS full scans were performed in the ultrahigh-field Orbitrap mass analyzer in ranges m/z 375–1500 with a resolution of 120 000 (at m/z 200). The top 20 most intense ions were subjected to Orbitrap for EV proteome analyses and top 30 for cell proteomes for further fragmentation via high energy collision dissociation (HCD) activation and a resolution of 15 000 with the AGC target set to 100%. We selected ions with charge state from 2+ to 6+ for screening. Normalized collision energy (NCE) was set at 30 and the dynamic exclusion to 40s.

1152 Data analysis: For identification, the data were searched against the *Homo Sapiens* (UP000005640) UniProt database using Sequest HT through proteome discoverer (version 2.4). Enzyme specificity was set to trypsin and a maximum of two miss cleavages sites were allowed. Oxidized methionine, Met-loss, Met-loss-Acetyl and N-terminal acetylation were set as variable modifications. Maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.02 Da for MS/MS peaks. The resulting files were further processed using myProMS v3.9.3⁹⁵ (<https://github.com/bioinfo-pf-curie/myproms>). FDR calculation used Percolator⁹⁶ and was set to 1% at the peptide level for the whole study. The label free quantification was performed by peptide Extracted Ion Chromatograms (XICs), reextracted across all conditions and computed with MassChroQ version 2.2.21⁹⁷. For protein quantification, XICs from proteotypic peptides shared between compared conditions (TopN matching) and missed cleavages were allowed. Median and scale normalization was applied on the total signal to correct the XICs for each biological replicate (N=5 for WT and N=10 for

1165 CD63 KO by combining two KO clones). To evaluate the statistical significance of the change
1166 in protein abundance, a linear model (adjusted on peptides and biological replicates) was
1167 performed, and a two sided T-test was applied on the fold change estimated by the model. The
1168 p-values were then adjusted using the Benjamini–Hochberg FDR procedure. Proteins with at
1169 least two distinct peptides in three replicates of a same state, a 2-fold enrichment and an adjusted
1170 p-value ≤ 0.05 were considered significantly enriched in sample comparisons. Proteins unique
1171 to a condition were also considered if they matched the peptides criteria. Proteins selected with
1172 these criteria were further subjected to Gene Ontology (GO) functional enrichment analysis as
1173 in⁹⁸ by using the complete gene ontology file. The mass spectrometry proteomics raw data have
1174 been deposited to the ProteomeXchange Consortium via the PRIDE⁹⁹ partner repository.
1175 Links for publicly accessible source data are listed in the Data Availability Statement and in
1176 Source Data files.

1177

1178 **Lipidomic analysis**

1179 Sample preparation: HeLa or MNT-1 cells were cultured in media supplemented with LPDS
1180 for 48 hours and sEVs were isolated by SEC as described above while cell pellets were
1181 resuspended in 100 μ l methanol.

1182 80 μ l of cell homogenate or total volume of EVs in suspension (150 μ l) was supplemented with
1183 a mixture of internal standards, and lipids were extracted with 700 (cells) or 800 (EVs) μ l
1184 methanol and 400 μ l chloroform in the presence of the antioxidant BHT and 310 μ l (cells) or
1185 160 μ l (EVs) of HCl 0,01N. Phase separation was triggered by addition of 400 μ l chloroform
1186 and 400 μ l water. Samples were vortexed for 1 min, centrifuged at 3600g for 10min at 4°C.
1187 Lower phase containing the lipids was dried and resuspended in 40 μ l of
1188 chloroform/acetonitrile/isopropanol (80:19:1 v/v). Quality controls (control serum independent
1189 of the study) were extracted in parallel of the samples.

1190 LC(HILIC)-MS/MS analysis of phospho- and sphingolipids: Phospho- (PL) and sphingolipids
1191 (SL) were quantified by LC-(ESI)MS/MS using a Prominence UFLC (Shimadzu, Tokyo,
1192 Japan) and a QTrap 4000 mass spectrometer (AB Sciex, Framingham, MA, USA).
1193 Quantification of phospholipids and sphingolipids was performed in positive-ion mode. Sample
1194 (4 μ l) was injected into a Kinetex HILIC column (150x2.1mm, 2.6 μ m) (Phenomenex, CA,
1195 USA). Mobile phases consisted of (A) 30mM ammonium acetate and 0.2% acetic acid and (B)
1196 acetonitrile supplemented with 0.2% acetic acid. Lipid species were detected in positive
1197 ionization mode using scheduled multiple reaction monitoring (sMRM) reflecting their head
1198 group fragmentation for PL and sphingomyelin (SM) (PIS (product ion scan) 184m/z for PC,

1199 LPC and SM, NL (neutral loss) 141m/z for PE, PIS (RCO+PE) for PE-P, NL 277m/z for PI,
1200 NL 185m/z for PS, NL 172m/z for LPG, NL (BMP head+R) for BMP, NL 189m/z for PG, NL
1201 115m/z for PA) and sphingoid base fragmentation for ceramides (PIS 266m/z and PIS 264m/z).
1202 Three independent injections were used: one injection for very minor lipids (PS, PA, LPC and
1203 LPE), one injection for the other lipids (PG, BMP, LPG, PE, PI, SM, ceramides, DHC), one
1204 injection for abundant lipids following sample dilution (PC and some SM).

1205 LC(C18)-MS/MS analysis of neutral lipids: Cholesteryl esters (CE) and free cholesterol were
1206 quantified by LC-(ESI) MS/MS using a Prominence UFLC and a QTrap 4000 mass
1207 spectrometer. Quantification of CE and cholesterol was performed in positive-ion mode.
1208 Sample (4µl) was injected into an Ascentis C18 column (150x2.1mm, 2.7µm). Mobile phases
1209 consisted of (A) acetonitrile/water (60:40 v/v) and (B) isopropanol/acetonitrile (90:10 v/v)
1210 supplemented with 10mM ammonium formate and 0.1% formic acid. Lipid species were
1211 detected using scheduled multiple reaction monitoring (sMRM) reflecting the fragmentation of
1212 the cholesterol core (PIS 369m/z).

1213 Data pre-processing: An in-house developed R script adapted from Ejsing et al¹⁰⁰ was used to
1214 correct for isotopic contribution to MRM signals in HILIC mode. Lipid features whose
1215 variability exceeded 30% in quality controls were discarded. Data were expressed normalized
1216 to total lipid signal (mol% of total lipids). This normalization was preferred for the statistical
1217 analysis as it allowed the comparison of cells and EV lipid composition and was independent
1218 of the size of the particles.

1219 Statistical Analysis: The statistical analysis of the lipidomics data were performed using Multi
1220 Experiment Viewer (MeV) statistical software package (version 4.9.0;
1221 <http://www.tm4.org/mev/>)¹⁰¹. Data are shown as mean ± SEM. Comparisons of the groups were
1222 performed by a 2 two-tailed Student's t-test. Features were considered significant when the p
1223 value was below 0.05 after Benjamini-Hochberg¹⁰² for false discovery rate (FDR) correction.

1224

1225 **Cholesterol levels measurement**

1226 Cells were grown in LPDS for 48h, EVs were isolated by SEC, and cells lysed. Amplex Red
1227 Cholesterol assay kit (Invitrogen, ThermoFisher Scientific) was used according to the
1228 manufacturer's protocol. Equal amount of EVs and cell lysate were loaded. For EVs,
1229 cholesterol content is reported as a ratio of the amount of cholesterol per EV.

1230

1231 **Alignment of tetraspanin sequences**

1232 Sequences of CD63 (P08962), CD81 (P60033), and CD9 (P21926) were obtained from Uniprot
1233 and alignment was performed using CLUSTAL O (1.2.4).

1234

1235 **3D structure of CD63 and interaction with cholesterol**

1236 A 3D structure of CD63 WT was predicted using the I-TASSER on-line server suite¹⁰³ by
1237 interrogating the server with CD63 (P08962) sequence for modelisation. Models were ranked
1238 based on their I-TASSER confidence (C) score (range 5 to +2 with a higher score correlating
1239 with an improved model). The model of prediction with the highest (C) score was selected
1240 (C=0,23).

1241

1242 **Molecular docking of cholesterol with CD63**

1243 The interaction between CD63 and cholesterol was modeled with Haddock 2.4 on-line server
1244 suite¹⁰⁴ by interrogating the server with the CD63 WT or E217Q 3D structures obtained from
1245 I-TASSER and a cholesterol molecule. CD63 residues were classified as active in cholesterol
1246 binding based upon CD81 residues previously implicated in cholesterol binding³⁴.

1247

1248 **Atomic force microscopy**

1249 *Sample preparation:* HeLa cells were cultured in media supplemented with LPDS for 48 hours
1250 and sEVs were isolated by SEC as described above.

1251 *AFM experiments:* AFM experiments and data analysis were performed as described in detail
1252 previously^{55,105}. sEVs were deposited on poly-L-lysine (PLL) coated glass coverslips prepared
1253 as follows. First, the coverslips were cleaned by immersing them in a 96% ethanol, 3%
1254 concentrated HCl solution for 10 min, and rinsed with ultrapure water. Then, coverslips were
1255 coated for 1 h in a 0.001 % poly-L-lysine (Sigma) solution, rinsed twice with ultrapure water,
1256 and dried at 37 °C overnight. The coated coverslips were stored at 7 °C and used for a maximum
1257 of 1 month. A PLL-coated coverslip was glued to a glass slide using conventional transparent
1258 superglue or two-component epoxy glue. After that, a glass ring was glued on top of the
1259 coverslip using JPK bio-compatible glue (Bruker) to hold the imaging buffer in place. The sEV
1260 sample was vortexed for ~5 s before 10 µl of sample were added to the center of the PLL-coated
1261 coverslip. After filling the chamber with PBS (imaging buffer), sEVs were imaged in QI mode
1262 on a JPK NanoWizard Ultra Speed setup situated on an inverted microscope (Olympus). The
1263 force set point during imaging was typically <100 pN, pixel time was set to 8.0 ms and z-length
1264 was set to 50-80 nm. Imaging was performed at room temperature (~22 °C). After imaging a
1265 single particle, the particle was indented at its center first with a maximum force of 0.4-0.5 nN,

1266 and then directly multiple times (typically 5 times) with higher forces (5-6 nN). The extend and
1267 retract speed of the cantilever during indentation was set to 0.3 $\mu\text{m/s}$ throughout all
1268 experiments. z-Length during indentation was 500 nm, sampling rate was set to 1024 Hz and
1269 the z-loop was closed. Both before and after indentation of the particle, the tip was checked for
1270 contamination and linear response by pushing it on the glass surface until a set force of 5 - 6
1271 nN was reached, recording a force distance curve. After indentation, another image was
1272 recorded to check for movement or collapse/changes of the particle. Only particles exceeding
1273 a minimum height (25 nm) were indented to avoid indenting stacks of multiple bilayers. Quartz-
1274 like cantilevers and tips were used with a nominal tip radius of <10 nm and a cantilever stiffness
1275 of 0.06 N/m, as specified by the manufacturer (qp-BioAC, Nanosensors). Individual cantilevers
1276 were calibrated using thermal tuning.

1277 AFM image analysis: Images were processed using JPK Data Processing Software. Cross
1278 sections were taken for each particle along the fast-scanning axis in order to analyze the shape
1279 and the size of the particle. The radius of curvature R_c was obtained by fitting a circular arc to
1280 the data above half of the maximum height of the spherical cap and correcting for the tip radius
1281 (10 nm as provided by the manufacturer). The height obtained from the fit was compared to the
1282 height obtained from the force indentation curve to ensure indentation at the particle's center.
1283 Recorded force distance curves were corrected for the offset using JPK Data Processing
1284 Software and subsequently exported for further analysis in Origin (version 2019). Assuming
1285 surface area conservation allowed us to calculate the radius R_0 of the particles in solution, as
1286 described previously^{55,105}.

1287 AFM FDC analysis: A linear fit was performed on the cantilever response measured on the
1288 PLL-coated glass coverslip. This result was used to transform the measured response into force
1289 indentation curves (FDCs) (see also¹⁰⁵). The contact point was found manually. By fitting a
1290 straight line to the data in the range 0.02–0.1 R_c the stiffness of the sEVs was obtained. For
1291 determining the tether force two lines with slope 0 were fit individually to the last detachment
1292 of the tip in the retraction curve before reaching the baseline again. The difference of the two
1293 intersects with the y-axis was calculated yielding the tether force. Only adhesion events
1294 exceeding the contact point were identified as tethers and were included. Tether forces > 0.25
1295 nN were excluded because these could correspond to multiple bilayer tethers, as reported
1296 previously⁵⁶. For fitting to the theory, described in detail elsewhere^{55,105}, the sum of the squared
1297 log Euclidian distance between the theoretical curve and the individual experimental data points
1298 was minimized by adjusting κ as a single fitting parameter. Confidence intervals were estimated
1299 using the bias corrected percentile method with 500 bootstrapping repetitions, for which a set

1300 of observed value combinations equal in size to the original data set was randomly drawn and
1301 fitted.

1302

1303 **EV uptake experiment**

1304 Producing cells were grown in LPDS and incubated with TF-chol for 16h at 1 uM. Fresh media
1305 (without TF-chol) was added and sEVs were prepared from conditioned media after 32h. EVs
1306 were isolated by UC and labeled or not with Membright 640 (Idylle, Lipilight) as previously
1307 described¹⁰⁶. Recipient cells were treated or not with U18666A overnight at 1 μM and fed with
1308 EVs from producing cells (considering a ratio of 5 producing cells for 1 recipient cell) for 24h
1309 hour before fixation and imaging with a confocal microscope Leica SP5 with a 63x 1.4 NA
1310 objective. Z-stack images were acquired every 0,18 μm.

1311

1312 **Image analysis and quantifications**

1313 ImageJ Fiji software (version 2.1.0/1.53c or 1.54F) was used.

1314 Manders' and Pearson's correlation coefficient

1315 Manders' and Pearson's correlation coefficients between two channels were quantified using
1316 JACoP plugin of ImageJ Fiji.

1317 Phalloidin fluorescence on endosomes

1318 Average intensity z projections of five stacks (acquired every 0.20 μm) were generated for both
1319 LAMP-1 or EEA1 and phalloidin channel. 15-pixel (1,65 nm)-diameter circles were drawn
1320 around LAMP-1- or EEA1-positive endosomes (ROI). The ROI was copied in the phalloidin
1321 channel and phalloidin mean fluorescence intensity in the ROI was measured using ImageJ Fiji.

1322 LDL-BODIPY, D4-GFP, pD4-mCherry, TF-chol, MemBright fluorescence intensity

1323 The mean intracellular fluorescence intensity or the integrated density was measured by
1324 manually selecting an area covering the entire cell with ImageJ Fiji.

1325 Filipin fluorescence intensity

1326 For each cell, the ratio of maximum intensity of fluorescence was measured by dividing the
1327 maximum intensity in U18666A treated cells compared to the DMSO treated cells. Data are
1328 presented as a percentage of enrichment of filipin intensity of fluorescence in U18666A treated
1329 cells compared to the DMSO control.

1330 Size, number, and distribution of TF-chol compartments

1331 Size and number of TF-chol compartments were measured by thresholding the intensity of
1332 fluorescence within the cells and analyzing particles with ImageJ Fiji. For the distribution of
1333 TF-chol compartments, ROI of interest were drawn around the nucleus and enlarged to locate

1334 TF-chol compartments within different areas of each cell from the perinuclear region to the
1335 plasma membrane. Data are presented as the percentage of perinuclear and peripheral
1336 compartments in the different conditions.

1337 Localization of internalized antibody directed against CI-M6PR

1338 Average intensity z projections of four stacks (acquired every 0,20 μm) were generated. The
1339 total intracellular fluorescence intensity was measured by manually selecting an area covering
1340 the entire cell. The fluorescence intensity within a $7 \times 7 \mu\text{m}^2$ region centered on the Golgi
1341 complex (TGN46 staining was used as reference) was then measured. The non-Golgi
1342 fluorescence (vesicular) intensity was obtained by subtracting the fluorescence intensity in the
1343 Golgi complex from the total fluorescence intensity. Data are presented as the non-Golgi/Golgi
1344 CIMPR fluorescence ratio from each cell.

1345 Immunogold labeling

1346 Quantification of immunogold labeling on ultrathin cryosections or EVs was performed by
1347 counting the number of gold particles per cell compartment or EV. MVEs were defined as
1348 compartments delimited by a membrane with numerous internal vesicles. The count was
1349 performed on randomly selected cells. As the D4-GFP staining can label free cholesterol that
1350 is not associated with membranes on EV samples, only the gold particles associated with EV
1351 membranes were quantified.

1352

1353 **Statistics & Reproducibility**

1354 The experiments were not randomized and the investigators were not blinded to allocation
1355 during experiments and outcome assessment. Experiments were performed multiple times, and
1356 experiments included in Figure 1a, 1b, 1c, 6d, Extended Data Fig. 1a, 4c, 4g were performed
1357 independently by two different investigators.

1358 No statistical method was used to predetermine sample size. No data were excluded from the
1359 analyses. For all assays, quantification and statistics were derived from $n= 3$ independent
1360 experiments, unless specified in the legends. For Fig. 4 b, Fig.5 d-f, Fig.6 f, g, Fig.7c, Fig. 8 a-
1361 c, ED Fig. 5g, ED Fig. 6c, ED Fig. 7 d, f, ED Fig. 8a, d, statistics were derived from $n=$ number
1362 of cells pooled across 2 independent experiments. Experiments in Fig. 4a and ED 5d were
1363 performed once and statistics were derived from $n=$ number of cells. Experiments shown in Fig.
1364 1f, 6e, ED Fig.1e, ED Fig.5a, ED Fig.7a, ED Fig. 8d, ED Fig.9 b,c are representative of 2
1365 independent experiments.

1366 Data are presented as mean \pm SEM, unless differently indicated in the legends. Statistics were
1367 calculated in GraphPad Prism 10 (version 10.2.1) using Student's t test, or Ordinary one-way

1368 ANOVA, or Kruskal-Wallis test or Mann-Whitney test, unless specified. Significant
1369 differences between control or treated samples are indicated (****, P <0.0001; ***, P <0.001;
1370 **, P < 0.01; *, P < 0.05, ns P < 0.05).

1371

1372 **Data availability**

1373 The mass spectrometry proteomics raw data have been deposited to the ProteomeXchange
1374 Consortium via the PRIDE partner repository. The datasets are publicly available at the
1375 following links: ProteomeXchange title: Human - Cells and Extracellular vesicles of HeLa and
1376 MNT-1 WT vs CD63 KO Proteomes 1, ProteomeXchange accession: PXD037146, Project
1377 Webpage: <http://www.ebi.ac.uk/pride/archive/projects/PXD037146> , FTP Download:
1378 <https://ftp.pride.ebi.ac.uk/pride/data/archive/2024/04/PXD037146>; ProteomeXchange title:
1379 Human - Cells and Extracellular vesicles of HeLa and MNT-1 WT vs CD63 KO Proteomes 2
1380 ProteomeXchange accession: PXD037147, Project Webpage:
1381 <http://www.ebi.ac.uk/pride/archive/projects/PXD037147>, FTP Download:
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1383 Human - Cells and Extracellular vesicles of HeLa and MNT-1 WT vs CD63 KO Exosomes
1384 ProteomeXchange accession: PXD037149, Project Webpage:
1385 <http://www.ebi.ac.uk/pride/archive/projects/PXD037149>, FTP Download:
1386 <https://ftp.pride.ebi.ac.uk/pride/data/archive/2024/04/PXD037149>.

1387 UniProt accession codes are: Homo Sapiens (UP000005640), P08962 for CD63, P60033 for
1388 CD81, and P21926 for CD9.

1389 Lipidomics datasets have been include in full in supplementary tables, provided in Source Data.
1390 All relevant data are included in the paper.

1391 Source data has been provided in Source Data. All other data supporting the findings of this
1392 study are available from the corresponding author on reasonable request.

1393

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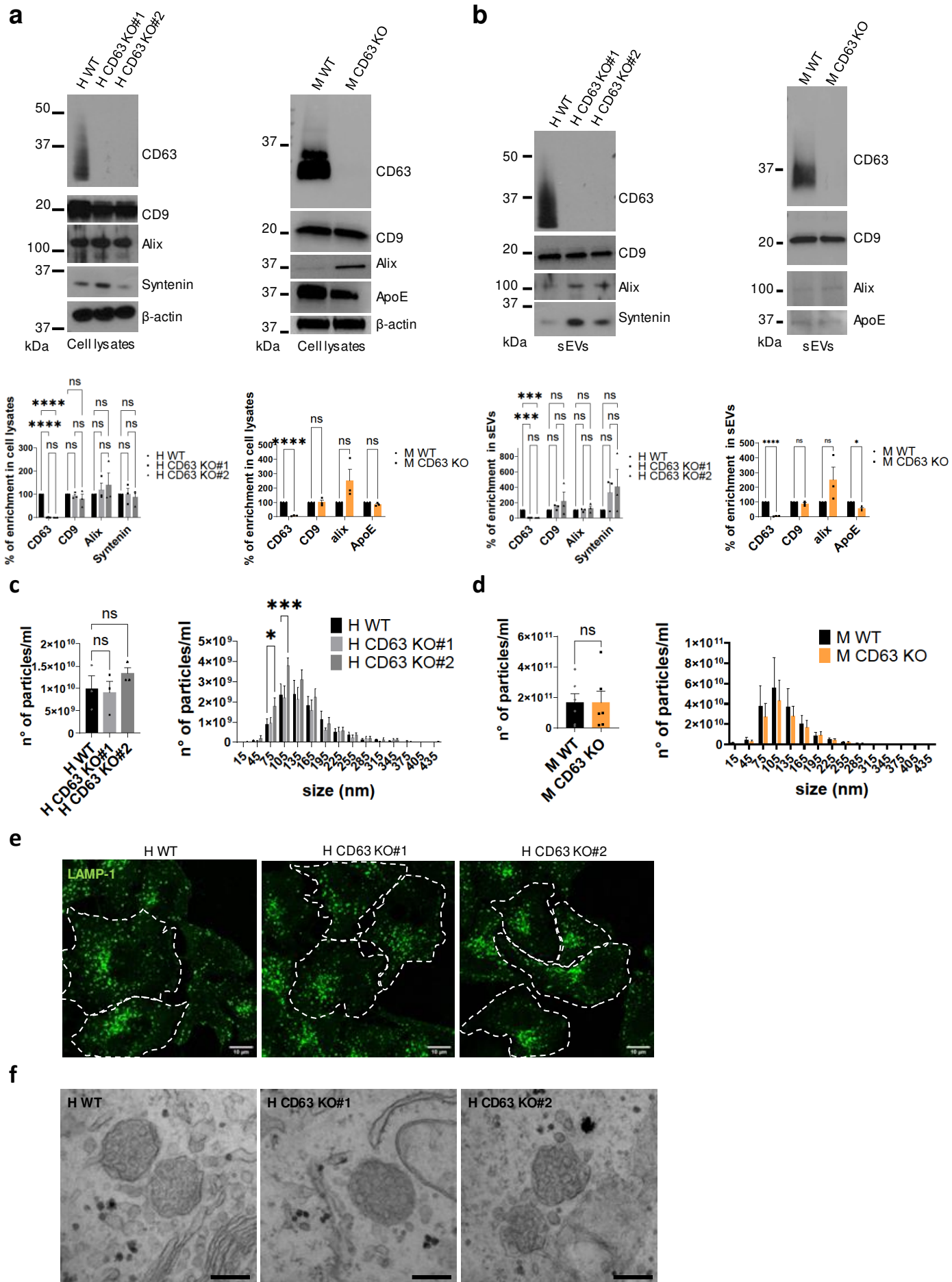
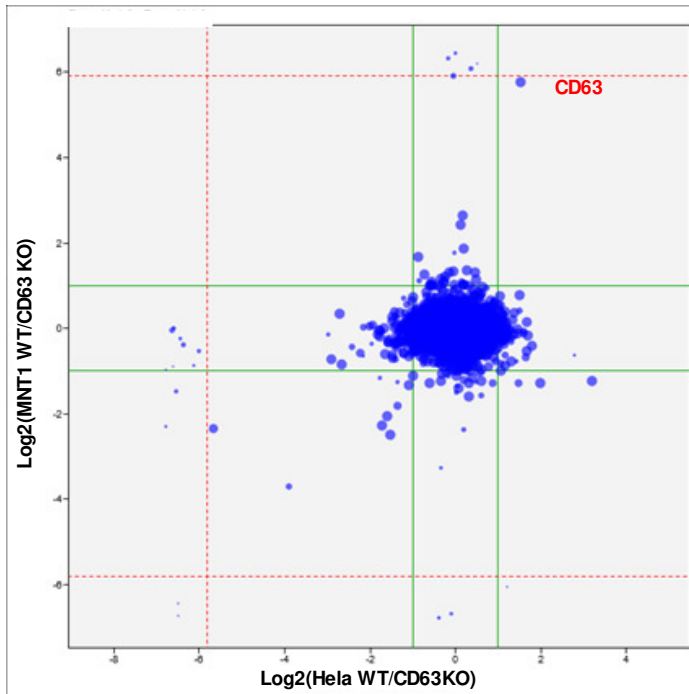
Fig. 1

Fig. 2

a

Correlation plot HeLa & MNT-1 proteomes

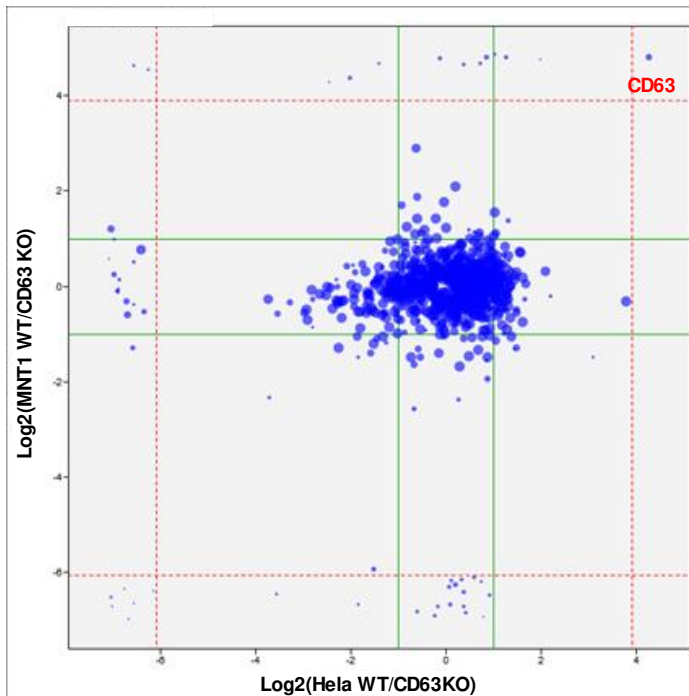


b

Protein Name	HeLa WT/CD63KO Ratio	MNT-1 WT/CD63 KO Ratio	MW (kDa)	Description
P08962	2.88	54.45	25.6	CD63 antigen

c

Correlation plot HeLa & MNT-1 sEVs



d

Protein Name	HeLa WT/CD63 KO Ratio	MNT1 WT/CD63 KO Ratio	MW (kDa)	Description
O15427	2.04	∞	49.5	Monocarboxylate transporter 4
P08962	∞	∞	25.6	CD63 antigen
P0CW18	2.04	2.93	64.6	Serine protease 56
P43003	2.40	∞	59.6	Excitatory amino acid transporter 1
Q9BY44	2.07	2.17	65.0	Eukaryotic translation initiation factor 2A

GO Term	Enrichment Factor	P-value
Symporter activity	54,767	5,22E-04
Carboxylic acid transmembrane transporter activity	49,6	6,36E-04
Organic acid transmembrane transporter activity	49,6	6,36E-04
Organic anion transmembrane transporter activity	36,511	1,17E-03
Secondary active transmembrane transporter activity	33,136	1,42E-03

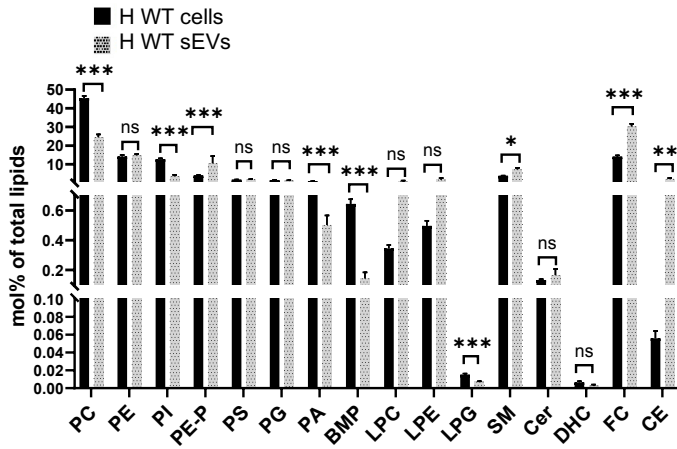
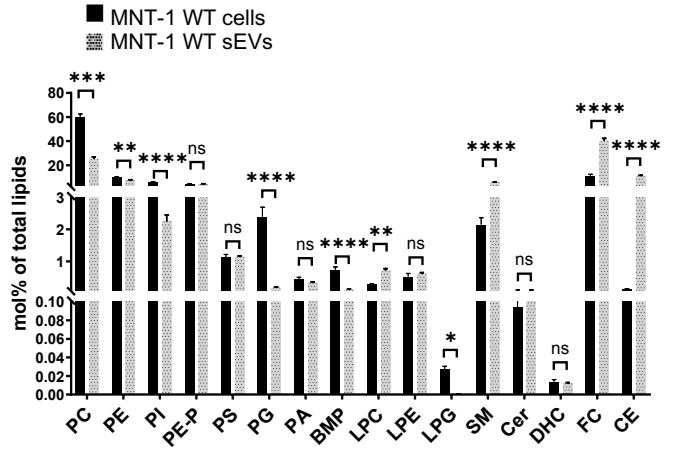
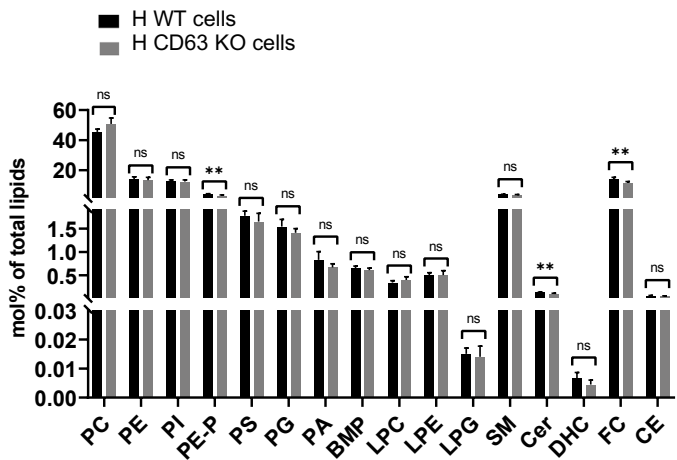
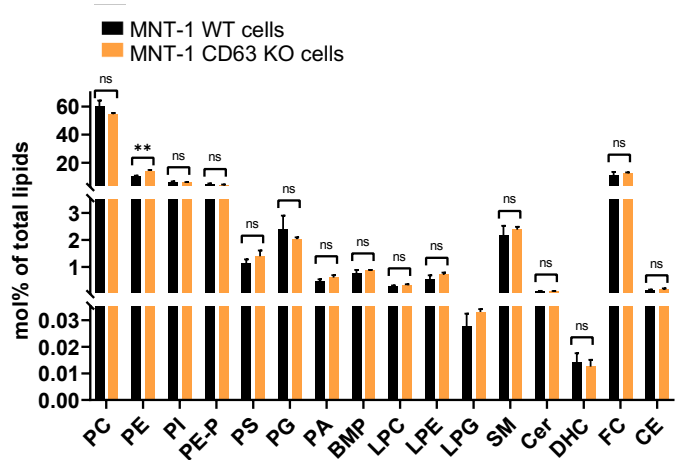
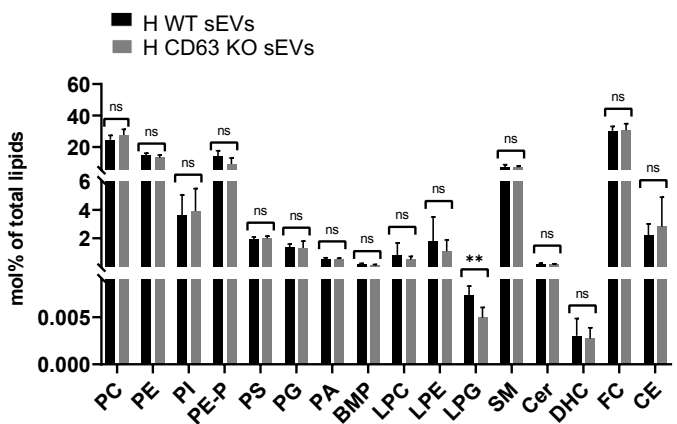
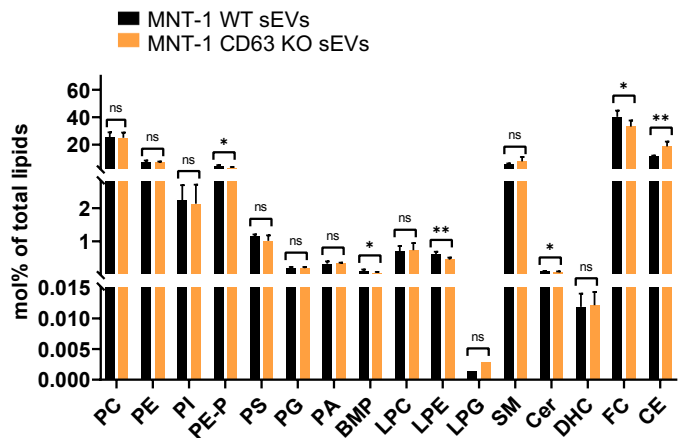
Fig. 3**a****b****c****d****e****f**

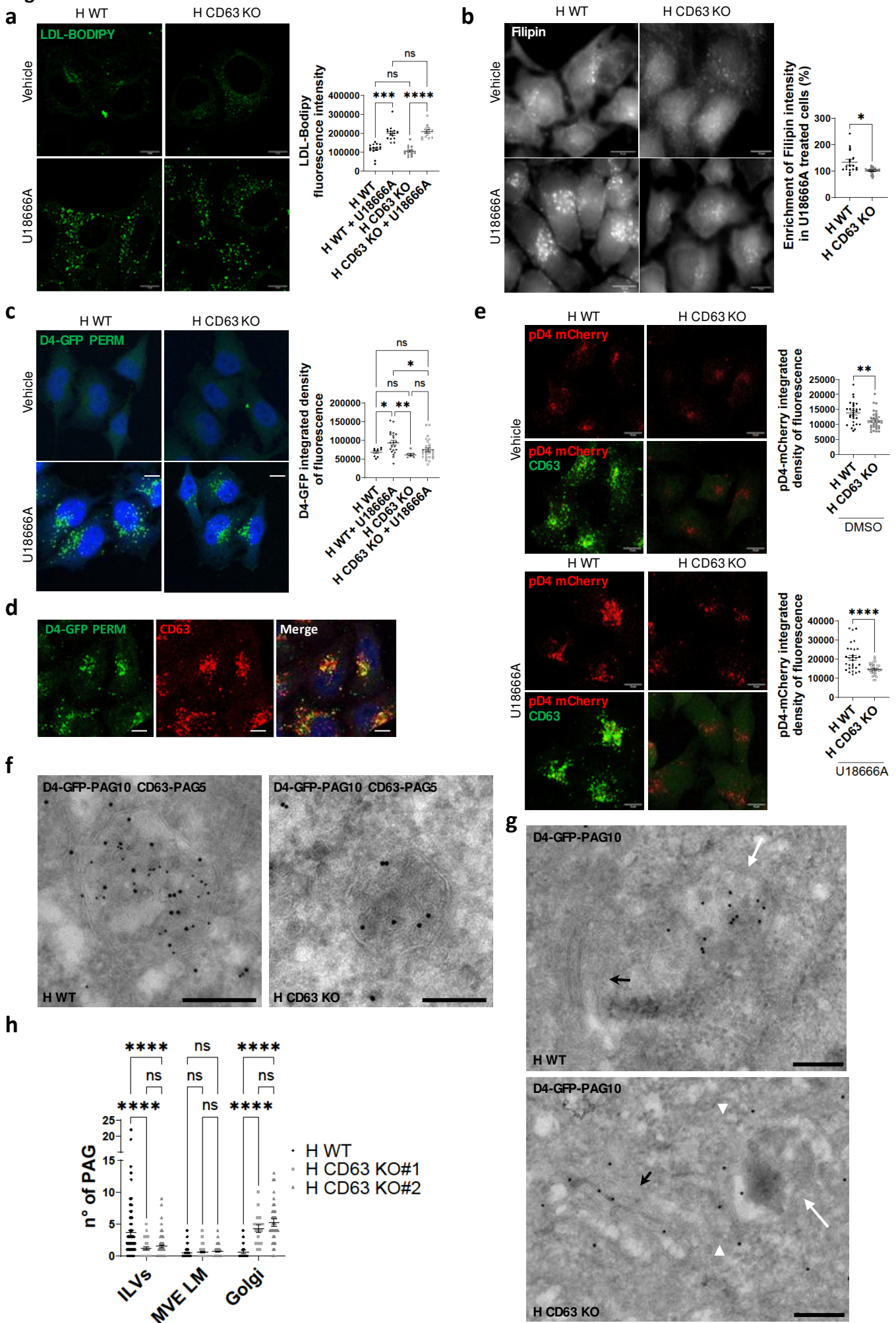
Fig. 4

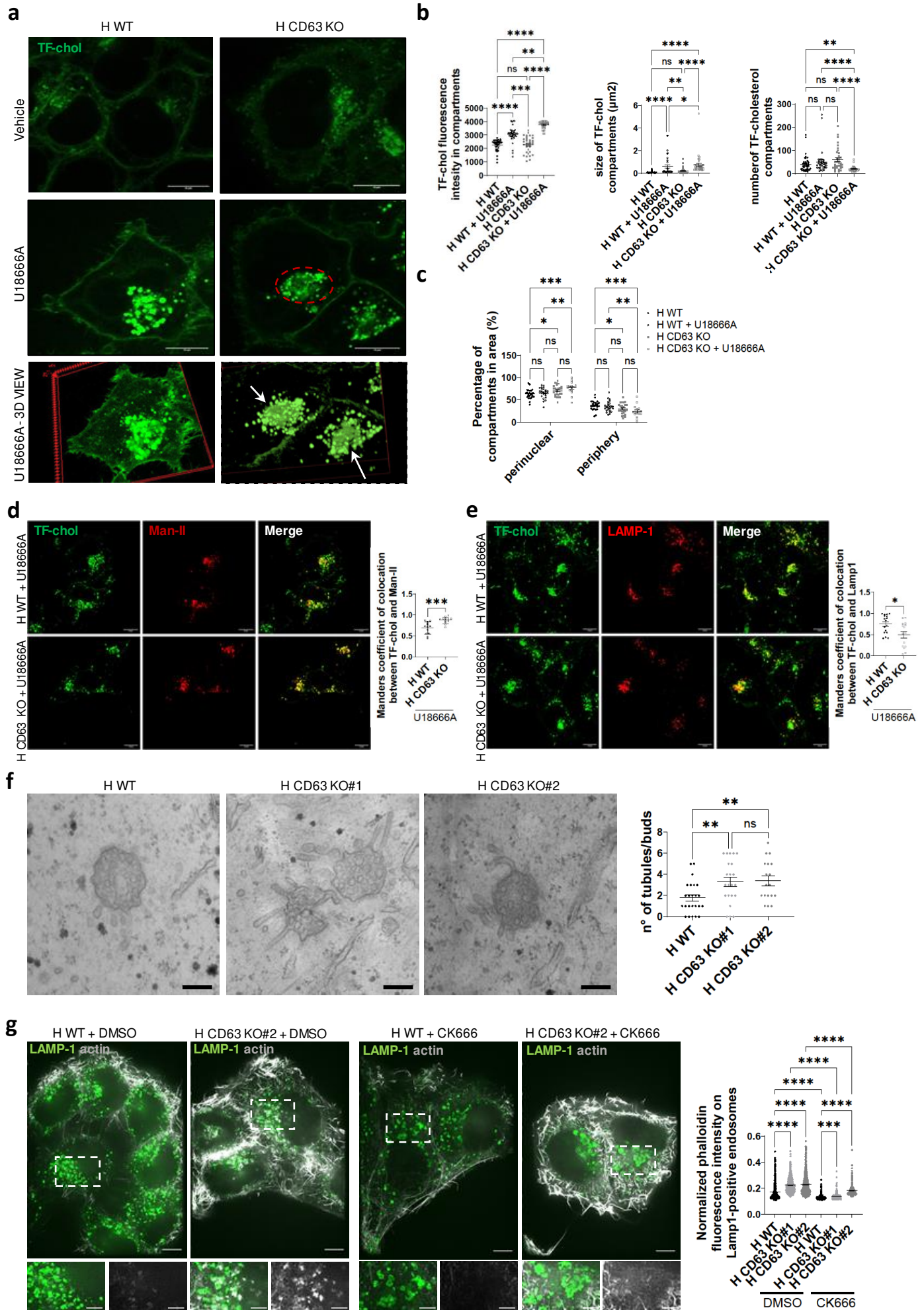
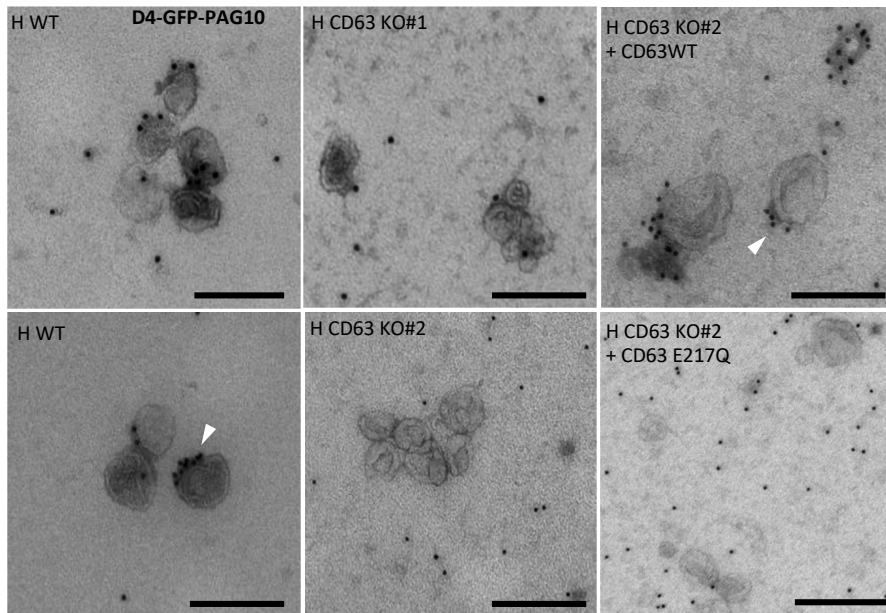
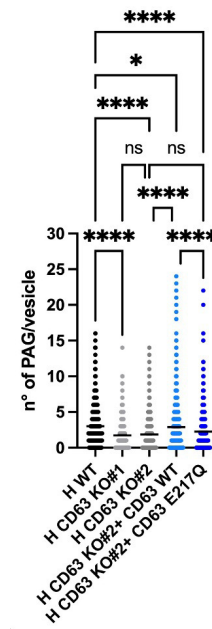
Fig. 5

Fig. 7

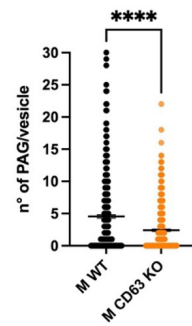
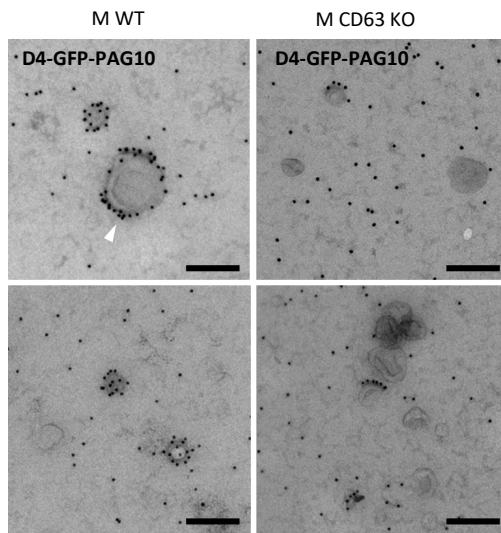
A



B



C



D

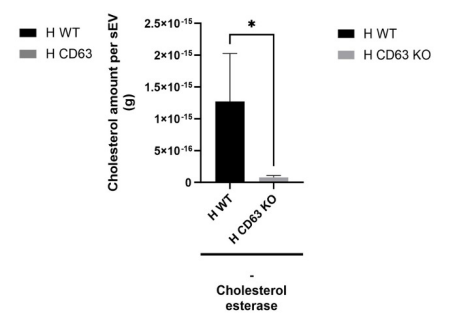
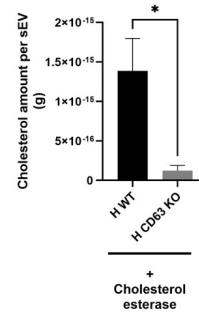
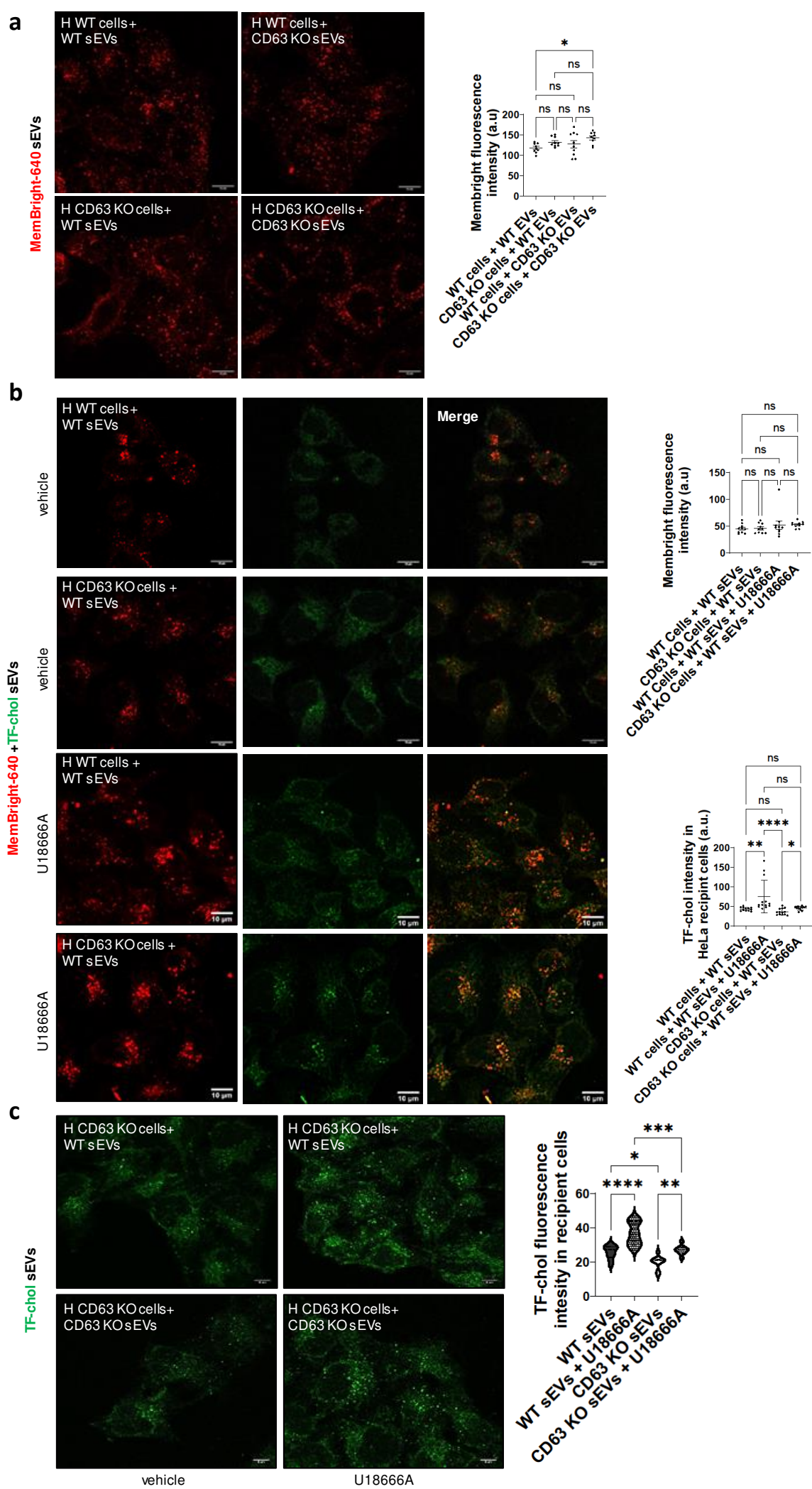
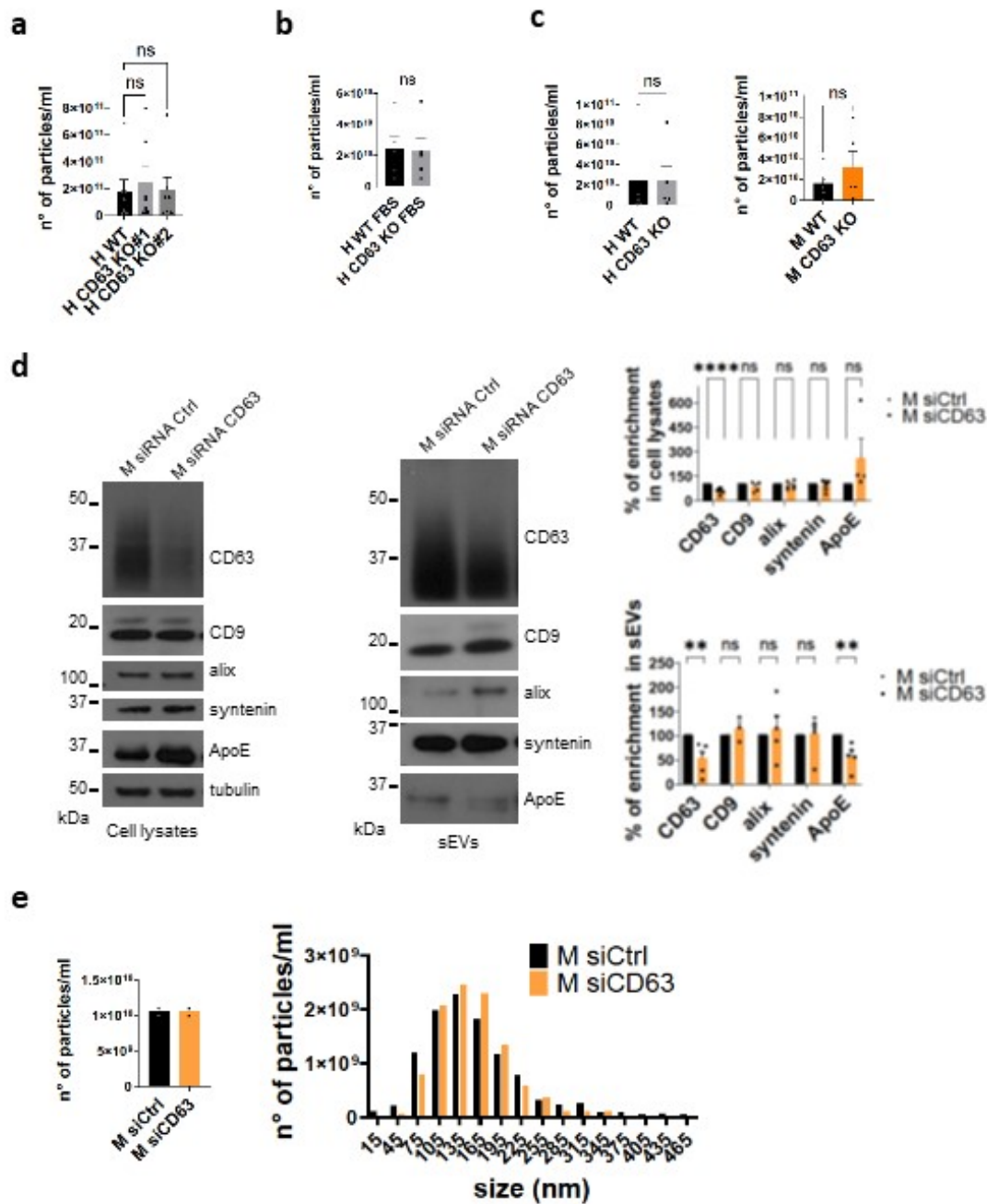


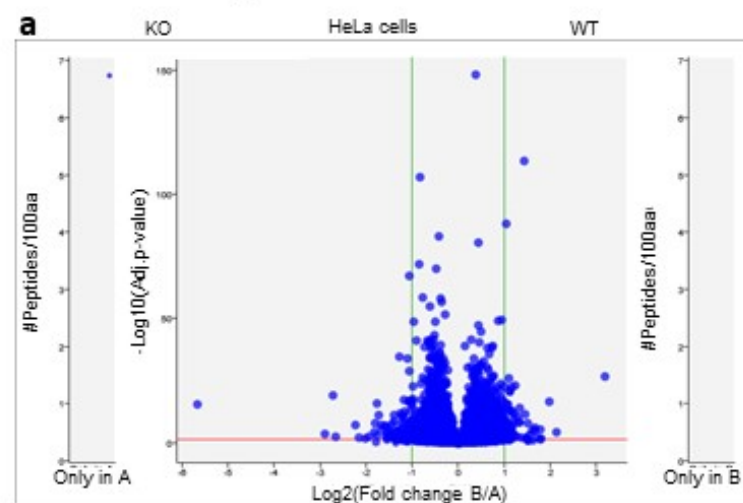
Fig. 8



Extended Data Fig. 1.



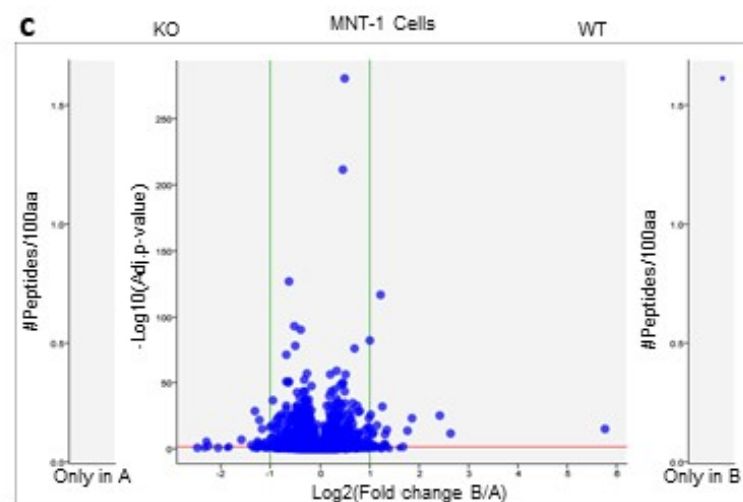
Extended Data Fig. 2



b

Name	Ratio	p-value	Description
WIPF1	9,16	1,74E-27	WAS/WASL-interacting protein family member 1
MEOX1	4,38	9,45E-05	Homeobox protein MOX-1
VTN	3,95	2,22E-17	Vitronectin
CPEB4	3,45	4,5E-06	Cytoplasmic polyadenylation element-binding protein 4
RAB6B	3,25	0,011094	Ras-related protein Rab-6B

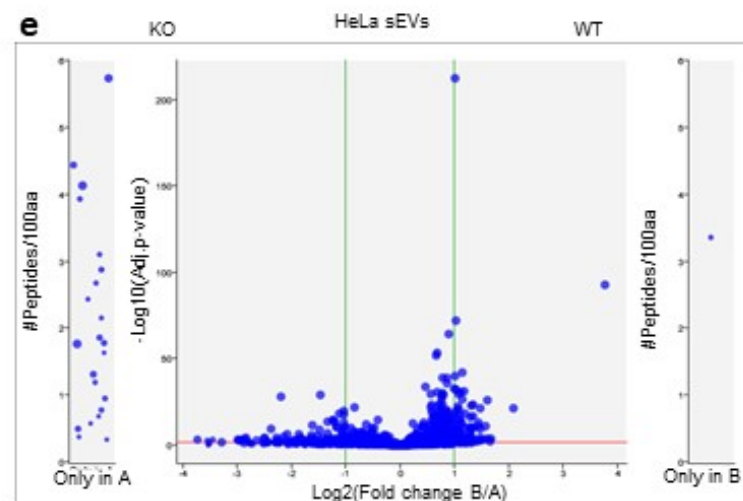
No Cellular components Go Terms found through analysis



d

Name	Ratio	p-value	Description
SDCCAG3	1000	=	Endosome-associated-trafficking regulator 1
CD63	54,45	1,11E-15	CD63 antigen
LGALS1	6,24	3,38E-12	Galectin-1
PRDX2	5,36	1,23E-25	Peroxiredoxin-2
ISYNA1	3,62	8,99E-24	Inositol-3-phosphate synthase 1

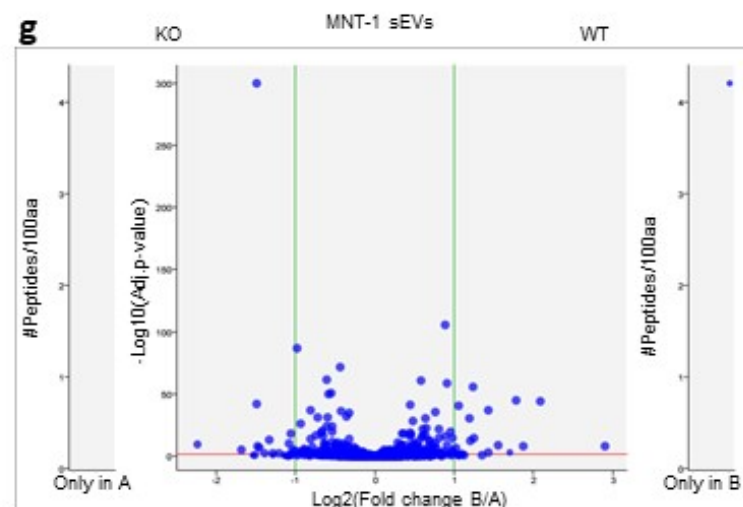
GO Term	Enrichment Factor	p-value
Membrane raft	12,27	4,60E-05
Membrane microdomain	12,231	4,67E-05
Membrane region	11,775	5,59E-05
Extracellular exosome	4,35	4,58E-06
Extracellular vesicle	4,307	5,09E-06



f

Name	Ratio	p-value	Description
CD63	1000	=	CD63 antigen
MMP12	13,67	1,91E-93	Macrophage metalloelastase
BASP1	4,25	1,27E-21	Brain acid soluble protein 1
RPL29	3,19	0,00019	60S ribosomal protein L29
STMN1	3,16	0,008814	Stathmin

GO Term	Enrichment Factor	P-value
Centralspindlin complex	144,44	6,30E-05
MPP7-DLG1-LIN7 complex	86,664	2,09E-04
Extrinsic component of external side of plasma membrane	54,165	5,79E-04
Eukaryotic translation initiation factor 4F complex	33,332	1,59E-03
Microvillus membrane	28,26	1,58E-04



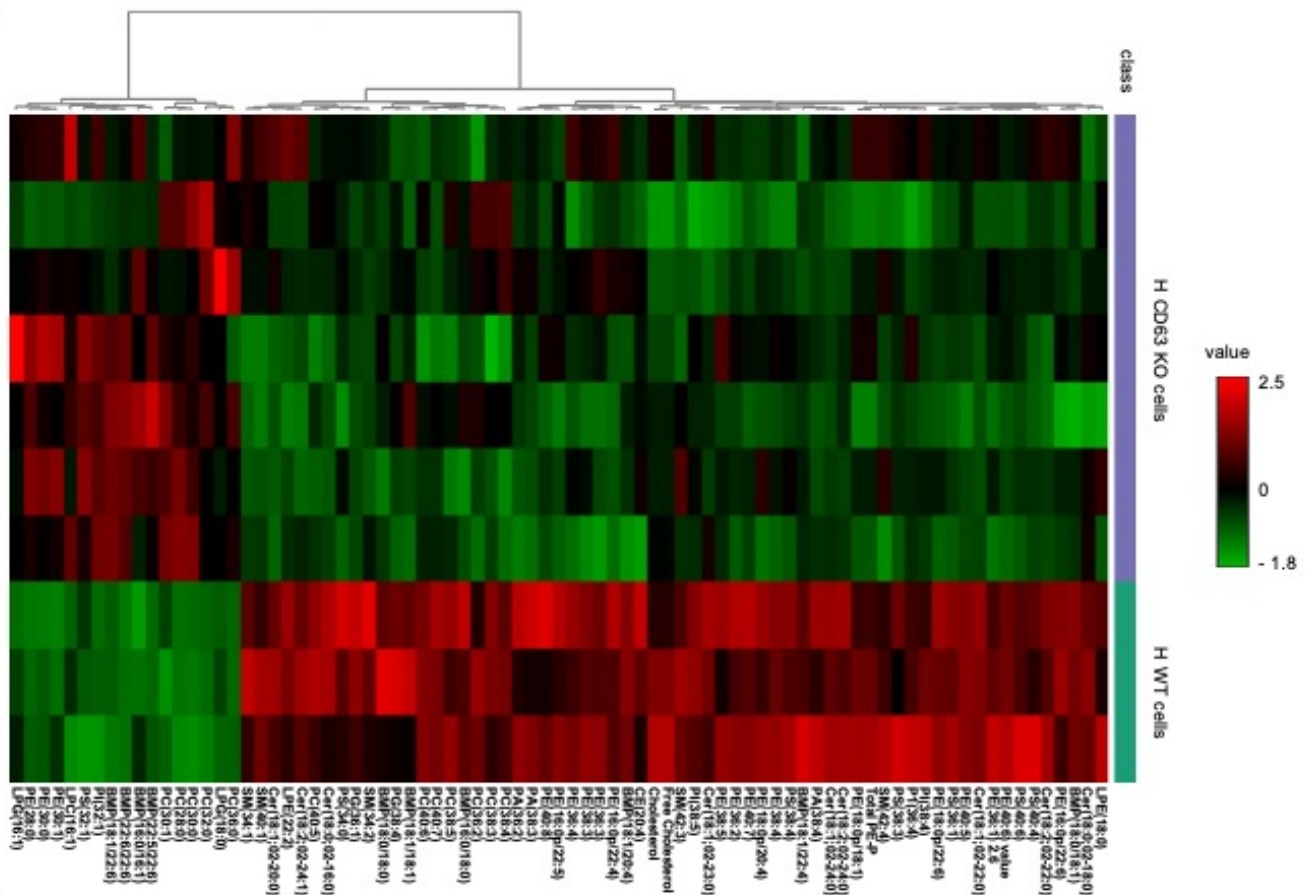
h

Name	Ratio	p-value	Description
CD63	1000	=	CD63 antigen
GPR56	7,49	6,75E-09	Adhesion G-protein coupled receptor G1
LGALS3BP	4,26	4,05E-45	Galectin-3-binding protein
VGf	3,65	8,08E-09	Neurosecretory protein VGf
PRELP	3,44	1,01E-45	Prolargin

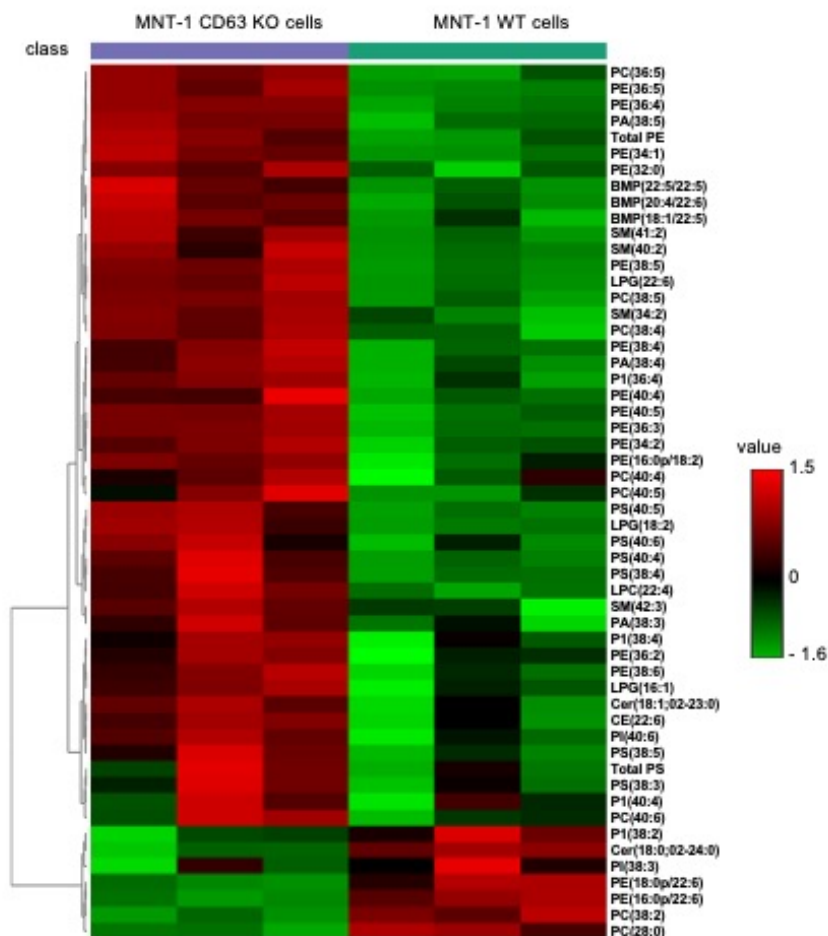
GO Term	Enrichment Factor	P-value
Mitochondria-associated ER Membrane	193,294	4,60E-05
Pigment granule membrane	165,681	6,33E-05
Chitosome	165,681	6,33E-05
Melanosome membrane	165,681	6,33E-05
Organelle membrane contact site	122,08	1,19E-04

Extended Data Fig. 3

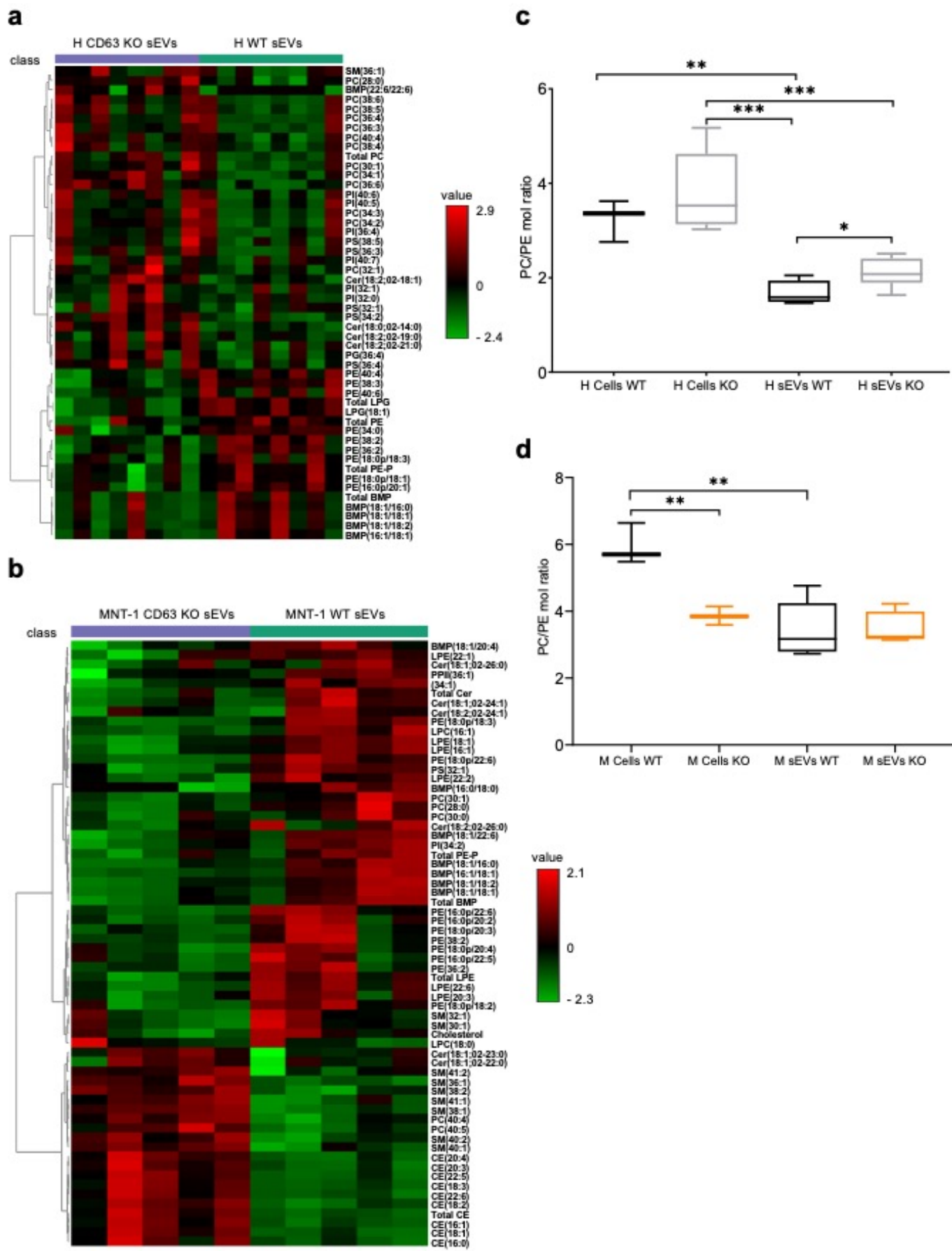
a



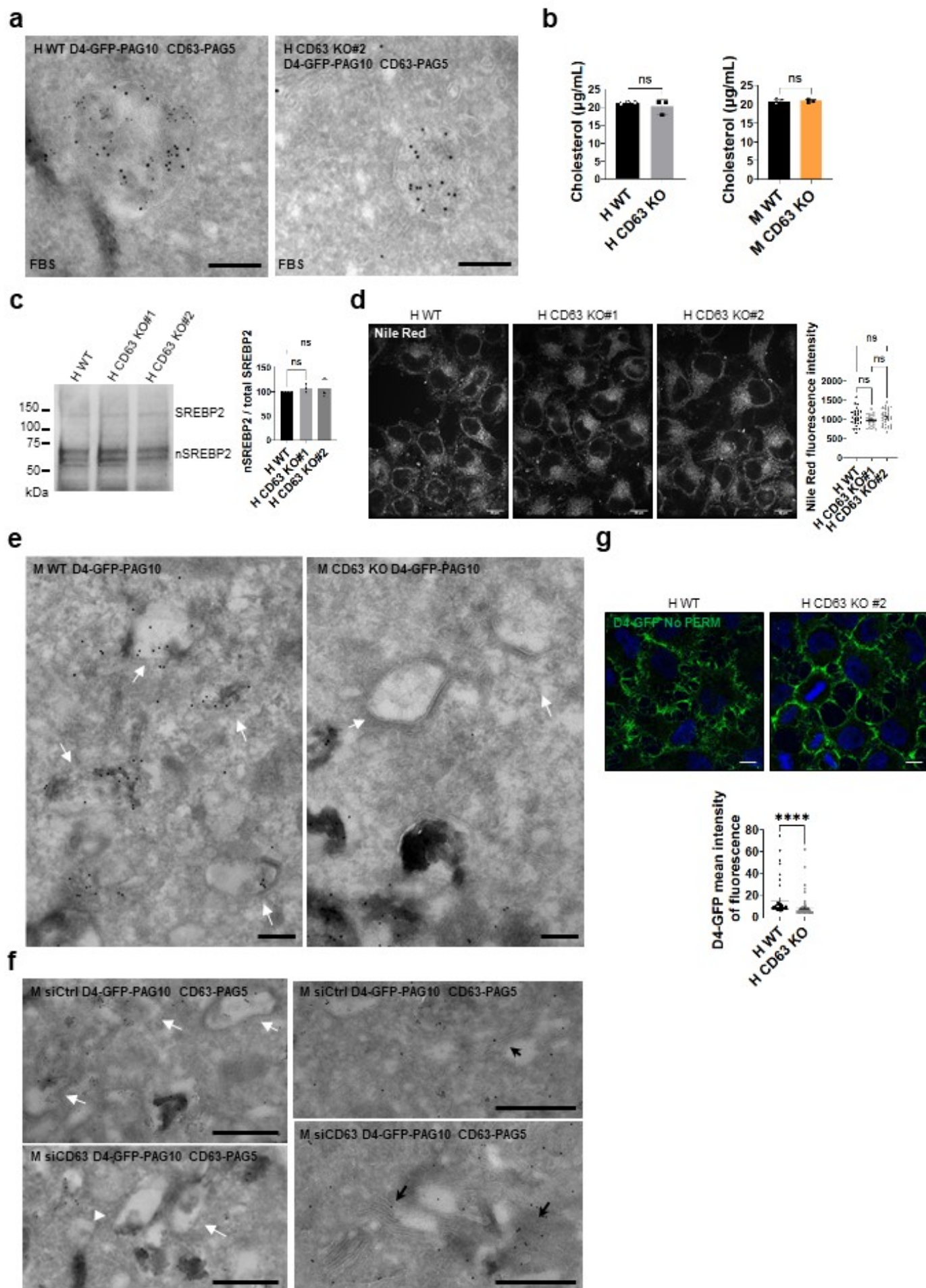
b



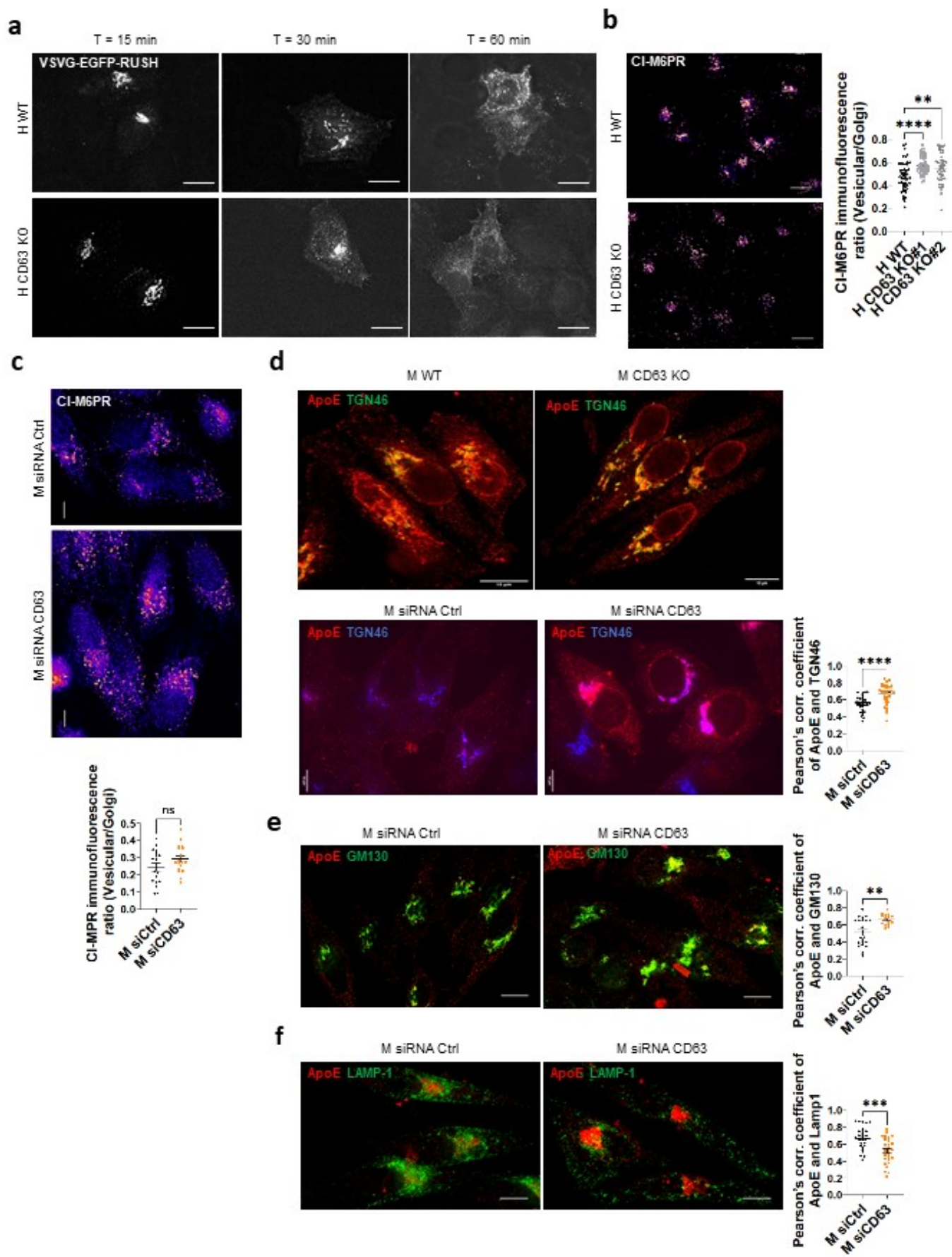
Extended Data Fig. 4



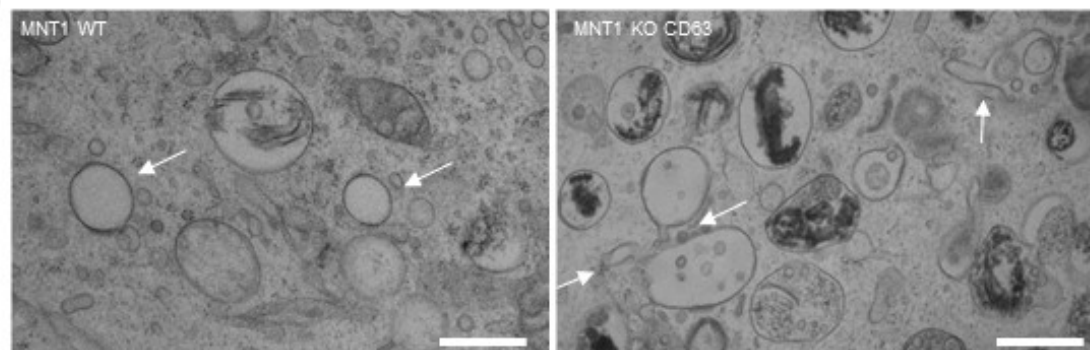
Extended Data Fig. 5



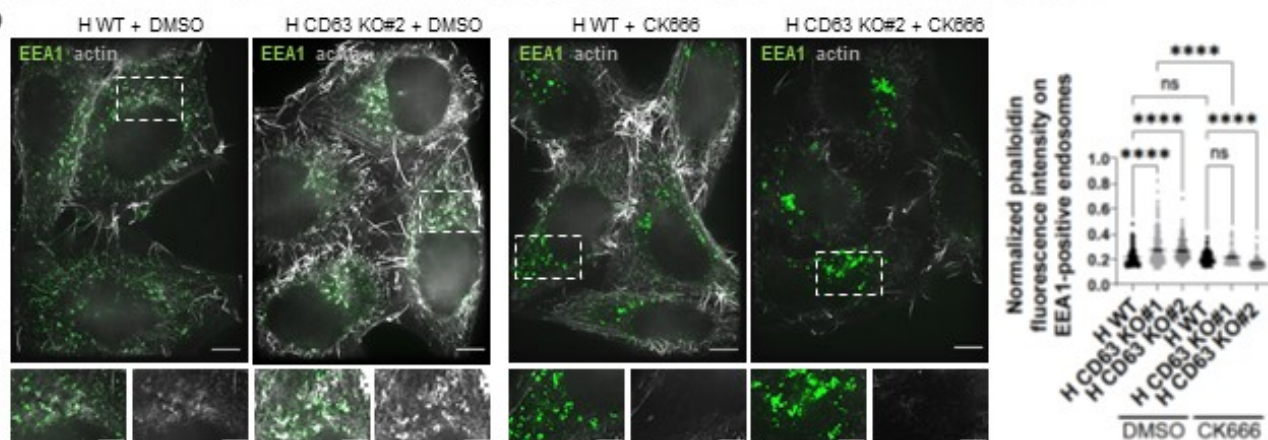
Extended Data Fig. 6



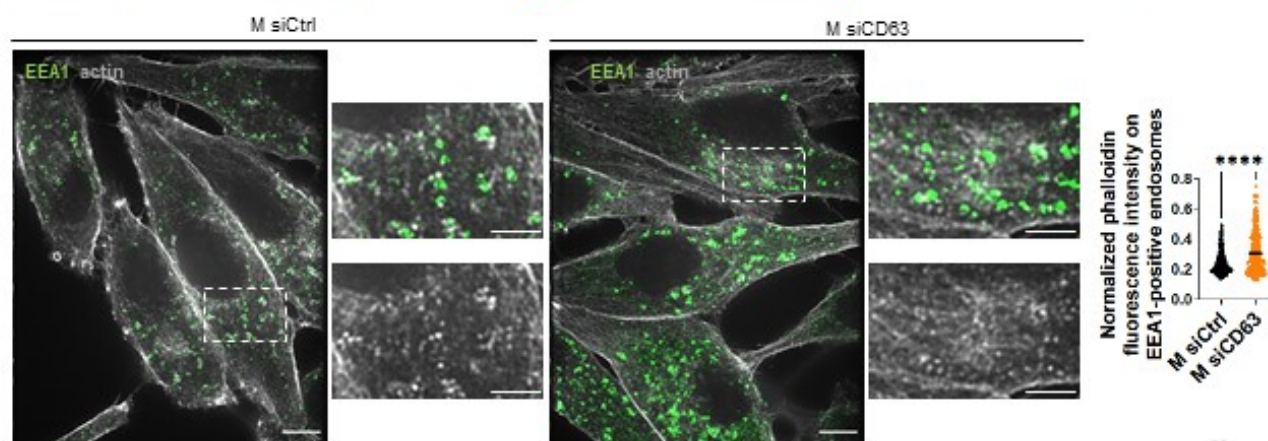
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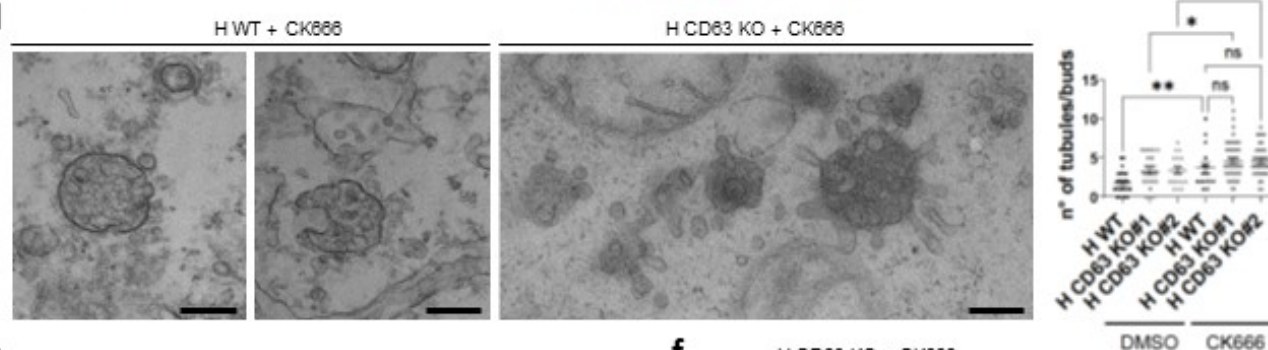
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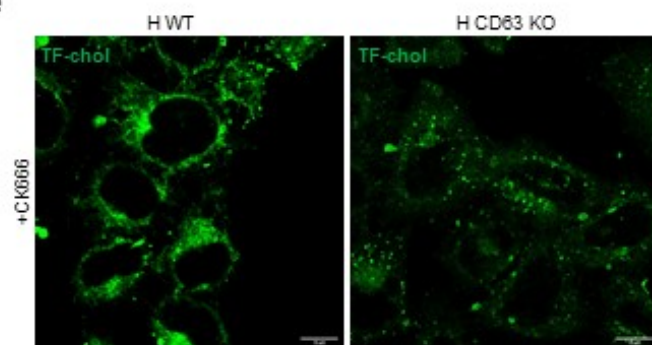
c



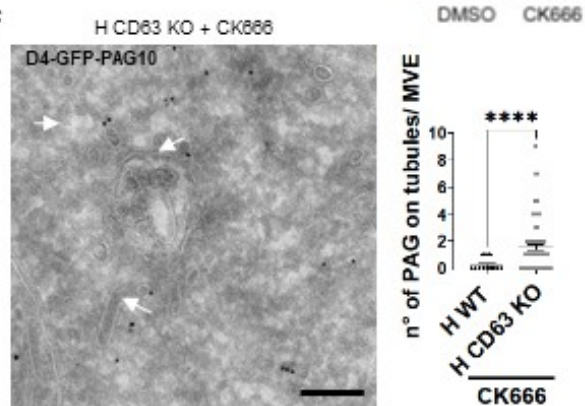
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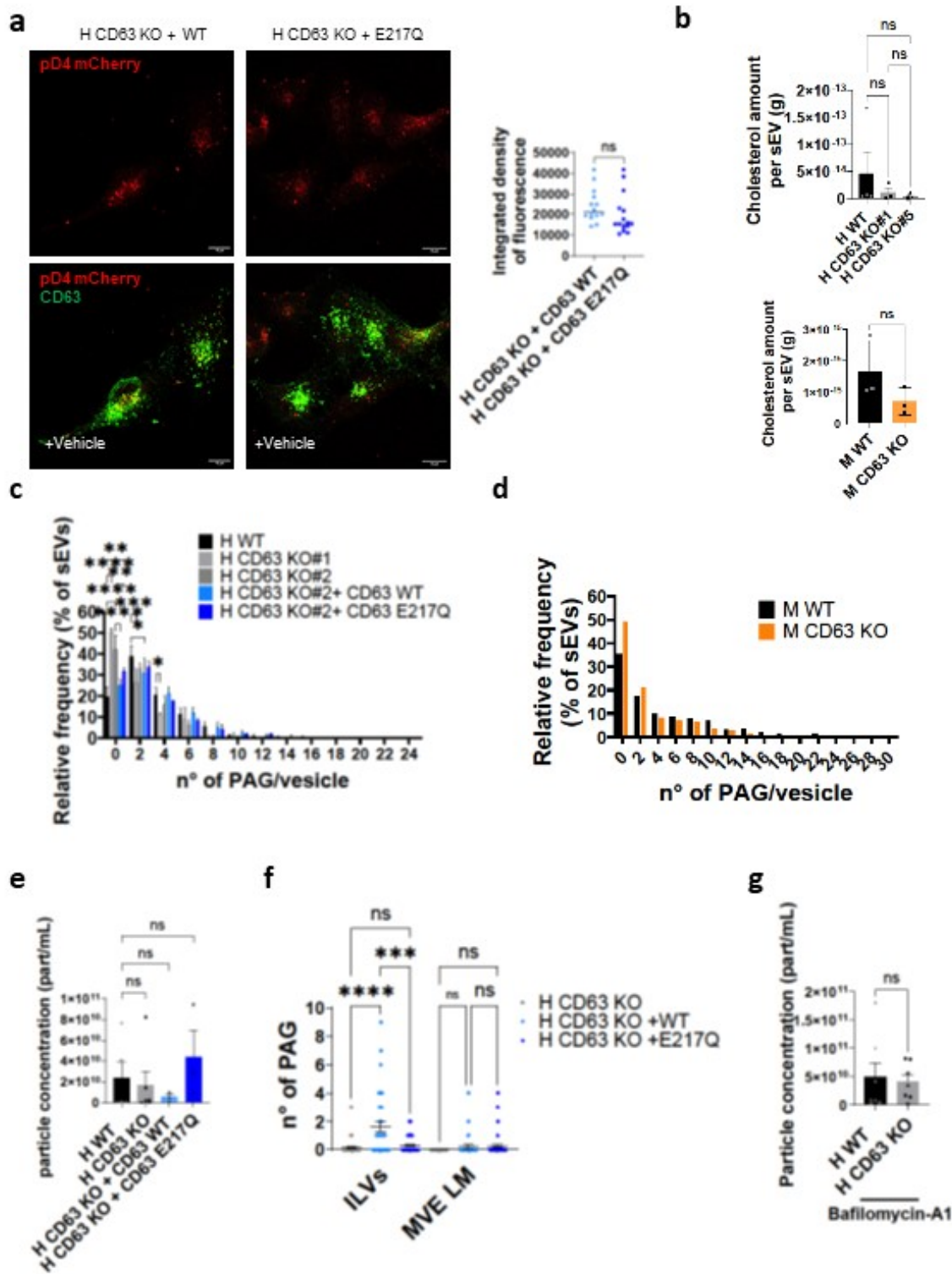
e



f



Extended Data Fig. 8



Extended Data Fig. 9

