

# 1 **Lipid accumulation promotes scission of caveolae**

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19 **ABSTRACT**

20 Caveolae, bulb-shaped invaginations of the plasma membrane (PM), show distinct behaviors  
21 of scission and fusion at the cell surface. Although it is known that caveolae are enriched in  
22 cholesterol and sphingolipids, exactly how lipid composition influences caveolae surface  
23 stability has not yet been elucidated. Accordingly, we inserted specific lipids into the PM of  
24 cells via membrane fusion and studied acute effects on caveolae dynamics. We demonstrate  
25 that cholesterol and glycosphingolipids specifically accumulate in caveolae, which decreases  
26 their neck diameter and drives their scission from the cell surface. The lipid-induced scission  
27 was counteracted by the ATPase EHD2. We propose that lipid accumulation in caveolae  
28 generates an intrinsically unstable domain prone to scission if not balanced by the restraining  
29 force of EHD2 at the neck. Our work advances the understanding of how lipids contribute to  
30 caveolae dynamics, providing a mechanistic link between caveolae and their ability to sense  
31 the PM lipid composition.

32 **SUMMARY**

33 Caveolae serve as mechanoprotectors and membrane buffers but their specific role in sensing  
34 plasma membrane lipid composition remains unclear. Hubert et al. show that cholesterol and  
35 glycosphingolipids accumulate in caveolae and drive subsequent scission from the cell  
36 surface. These results provide new insight into how lipids contribute to budding and scission  
37 of membrane domains in cells.

## 38 INTRODUCTION

39 Caveolae are bulb-shaped invaginations of the plasma membrane (PM), enriched in  
40 cholesterol (Chol), sphingolipids and the integral membrane protein caveolin1 (Cav1) (Parton  
41 & del Pozo, 2013). Caveolae are present in most cell types, with a particularly high density in  
42 endothelial cells, adipocytes and smooth muscle cells. Absence or malfunction of caveolae is  
43 associated with a number of conditions such as lipodystrophy, muscular dystrophy and  
44 cardiovascular diseases (Cohen et al., 2004; Pilch & Liu, 2011). Whilst the mechanism of  
45 how caveolae dysregulation drives the phenotype of disease is not well understood, they have  
46 been proposed to serve as signaling platforms, endocytic carriers, and PM reservoirs involved  
47 in mechanoprotective processes or lipid buffering (Parton & del Pozo, 2013; Sinha et al.,  
48 2011). In adipocytes, which are key lipid homeostasis regulators, caveolae are estimated to  
49 account for more than 50% of the surface area (Thorn et al., 2003). The clinical manifestation  
50 of caveolae loss in both patients (Cao et al., 2008; Hayashi et al., 2009; Kim et al., 2008) and  
51 mouse models (Liu et al., 2008; Razani et al., 2002) reveals severe malfunction of adipocytes,  
52 in addition to other cell types involved in lipid turnover and storage.

53 Biogenesis of caveolae is tightly coupled to the PM lipid composition and is thought to be  
54 driven by Chol-sensitive oligomerization of Cav1 and subsequent association with the cavin  
55 coat proteins (Fig. S1A) (Parton & del Pozo, 2013). Cav1 is embedded into the lipid bilayer  
56 via its intramembrane and scaffolding domains, which interact with Chol (Parton & del Pozo,  
57 2013). Chol depletion from the PM causes caveolae disassembly, leading to the  
58 disassociation of cavins from Cav1, which then disseminates throughout the PM (Morén et  
59 al., 2012; Rothberg et al., 1992). Relative to the bulk PM, phosphatidylserine,  
60 glycosphingolipid (GSL) and sphingomyelin (SM) has also been proposed to be enriched in  
61 caveolae (Hirama et al., 2017; Örtengren et al., 2004; Singh et al., 2010). However, it has not  
62 been determined whether specific lipids are enriched in caveolae in living cells and,

63 furthermore, how exactly they influence biogenesis, *i.e.*, through a general physical effect on  
64 the membrane bilayer or via direct interactions with the caveolae coat proteins. Additionally,  
65 it is not known whether these lipids can diffuse freely in and out of the caveolae bulb or if  
66 they are sequestered by interactions with the caveolae coat and membrane curvature  
67 restraints.

68 Whilst caveolae are typically associated with the PM as bulb-shaped invaginations, they also  
69 exhibit dynamic behaviour including flattening (Nassoy & Lamaze, 2012), short-range cycles  
70 of fission and fusion with the PM, and endocytosis (Boucrot et al., 2011; Morén et al., 2012;  
71 Pelkmans & Zerial, 2005). Caveolae are stabilized at the cell surface by the ATPase Eps-15  
72 homology domain-containing protein 2 (EHD2), which oligomerizes around the neck of  
73 caveolae, restraining their scission (Fig. S1A) (Morén et al., 2012; Stoeber et al., 2012).  
74 EHD2 extensively colocalizes with Cav1 and most of the membrane-associated EHD2 is  
75 found in caveolae (Morén et al., 2012). Although not considered part of the caveolae coat,  
76 EHD2 appears to be a critical component for maintaining caveolae integrity in terms of  
77 surface attachment. Despite that all the above-mentioned proposed functions of caveolae  
78 would heavily depend on whether they are surface associated or released, it is not clear how  
79 the balance between these states is controlled physiologically. Furthermore, the biological  
80 function of their atypical dynamics remains elusive.

81 Lipids are also thought to influence caveolae dynamics, *e.g.*, addition of bovine serum  
82 albumin-complexed lactosyl ceramide (LacCer) and elevated levels of Chol have been  
83 proposed to reduce the number of surface-connected caveolae and increase their mobility (Le  
84 Lay et al., 2006; Sharma et al., 2004). However, despite their essential structural role in  
85 caveolae, little is known about how they influence caveolae biogenesis and dynamics. This  
86 knowledge gap can be partly attributed to limitations associated with the current methods

87 employed to study such phenomena. Drugs such as statins, that inhibit Chol synthesis, require  
88 multi-day treatments, and, in addition to altering transcriptional regulation, may also elicit  
89 major secondary effects (Crescencio et al., 2009). This results in downregulated expression of  
90 Cav1, making it difficult to decipher between the effects of Chol levels on caveolae  
91 biogenesis and caveolae dynamics. In addition, drugs such as myriocin, which inhibit  
92 sphingosine synthesis, will also affect the levels of all sphingolipid species, thus hampering  
93 direct conclusions. BSA-coupled Bodipy-LacCer has been used as a fluorescent marker of  
94 endocytosis (Puri et al., 2001; Sharma et al., 2004; Singh et al., 2006; Singh et al., 2003), but  
95 this procedure involved PM loading at 4°C followed by a temperature shift to 37°C, known to  
96 heavily influence membrane fluidity and exacerbate endocytosis (Kleusch et al., 2012).  
97 While previous work indicates that lipids might influence both caveolae numbers and their  
98 dynamics, they have been unable to address whether caveolae dynamics respond directly to  
99 alterations in PM lipid composition and if proposed effects are dependent on concentration or  
100 species of different lipids present. In general, our understanding of the levels of quantitative  
101 changes in PM lipid composition that can be sensed and controlled is relatively sparse, not to  
102 mention the alteration in lipid composition required to influence caveolae. It is also not  
103 known if lipids could affect caveolae dynamics by changing the composition of the caveolae  
104 bulb or the surrounding membrane and whether the proposed effects on caveolae mobility is  
105 caused by direct effects on caveolae scission from the cell surface.

106 To address this, we aimed to rapidly and selectively manipulate cellular membrane lipid  
107 composition in a system where both the lipids and caveolae could be tracked. Here, we have  
108 applied fusogenic liposomes that allowed us to directly insert specific unlabeled or  
109 fluorescently-labeled lipids into the PM of living cells and study their effect on caveolae  
110 dynamics. Our data shows that a relatively small increase in glycosphingolipids and Chol  
111 results in their accumulation in caveolae, reducing the caveolae neck diameter, and driving

112 caveolae scission from the PM. EHD2 was identified to counterbalance the stability of  
113 caveolae in response to lipid composition and in accordance with a recent study (Matthäus et  
114 al., 2019; Morén et al., 2019), we describe a key regulatory role of EHD2 in lipid  
115 homeostasis.

## 116 **RESULTS**

### 117 **Lipids rapidly insert into the PM of living cells via liposome-mediated membrane fusion**

118 As a tool to study the effects of an altered lipid composition on caveolae dynamics, we  
119 employed fusogenic liposomes. This enabled us to rapidly insert lipids into the PM of HeLa  
120 cells via membrane fusion (Fig. 1A). To assess the effect of lipids known to be enriched in  
121 caveolae, different Bodipy-labeled analogues of sphingolipids (Cer, SM C<sub>5</sub> and SM C<sub>12</sub>),  
122 GSLs [ganglioside GM1 and lactosyl ceramide (LacCer)], Chol and phosphatidyl  
123 ethanolamine (PE) (Fig. S1B) were incorporated into liposomes [DOPE/Dotap/Bodipy-  
124 tagged lipid (47.5/47.5/5)] (Csiszar et al., 2010). Liposomes had diameters between 160-300  
125 nm (Fig. S1C) and an average fluorescence per liposome of 600 a.u (Fig. S1D). The Bodipy  
126 fluorophore allowed us to track and quantify the lipid incorporation in the PM and study  
127 colocalization with caveolae components. To ensure the observed effects are not significantly  
128 influenced by the fluorophore motif, the results were verified with unlabeled lipids. Fusion of  
129 the liposomes with the PM of HeLa cells occurred immediately upon contact and the lipids  
130 were rapidly distributed throughout the basal membrane, as observed using live-cell TIRF  
131 microscopy (Figs. 1B and S1E, exemplified by LacCer). The total fluorescence attributed to  
132 the Bodipy motif increased uniformly in various regions of interest (ROIs) (Fig. 1B, bar plot).

133 To determine the amounts of lipids that were incorporated in the membrane through liposome  
134 fusion, we used quantitative mass spectrometry on whole cells as 90% of these lipids are  
135 located in the PM (Lorizate et al., 2013). The method was verified by altering the lipid

136 composition using myriocin (24h treatment) or sphingomyelinase (SMase, 2h treatment),  
137 which are known to lower the levels of sphingomyelin (Gulshan et al., 2013). Analysis  
138 showed that these treatments drastically decreased SM(d18:1/16:0) levels, the major  
139 endogenous species of SM (Fig. 1C). Next, we incubated cells with fusogenic liposomes  
140 containing Bodipy-labeled LacCer or SM C<sub>12</sub>, and analyzed the lipid composition by LC-  
141 ESI-MS/MS. The detected levels of endogenous LacCer(d18:1/16:0) and SM(d18:1/16:0) in  
142 untreated control samples were in agreement with previously reported levels in HeLa cells  
143 (Kjellberg et al., 2014). In comparison, in samples treated with fusogenic liposomes, the  
144 incorporated Bodipy-lipids could be detected (Figs. S1F-G). The incorporated levels of  
145 Bodipy-LacCer and Bodipy-SM C<sub>12</sub> per 400 000 cells were measured to be 4.2 pmol and 2.7  
146 pmol, respectively, (*i.e.*,  $6.3 \times 10^6$  and  $4.0 \times 10^6$  lipids/cell) (Fig. 1D). To assess the  
147 incorporation efficiency of Chol, deuterium labeled Chol, d7-Chol, was included into  
148 fusogenic liposomes. GC-MS/MS analysis revealed that d7-Chol was incorporated to similar  
149 levels as Bodipy-labeled LacCer and SM C<sub>12</sub>. Given that the PM of these HeLa cells harbor  
150 around  $7 \times 10^9$  lipids/cell (see Methods section for details), the levels of Bodipy- and d7-  
151 labeled lipids detected by mass spectrometry led to a 0.02-0.09% increase in specific labeled  
152 lipids and a 0.4-1.6% of total lipids.

153 To determine the rate of incorporation of the different Bodipy-labeled lipid species, we used  
154 spinning disk microscopy in a central confocal plane of the cell (Fig. 1E). Quantitative  
155 analysis of lipid incorporation into the PM over time revealed similar levels for most lipids  
156 ranging from 1900 to 4800 arbitrary units at 10 min (Fig. 1E). The variation in incorporation  
157 rates between the different lipids may be due to marginal differences in the fusogenicity of  
158 the liposomes or differences in the PM-turnover of each particular lipid. To monitor the  
159 lateral diffusion of the Bodipy-lipids within the PM, cells were incubated with fusogenic  
160 liposomes for 10 min, and the recovery of Bodipy fluorescence in a defined region of interest



161 (ROI) after bleaching was monitored. Lateral diffusion within the PM was similar between  
162 the different Bodipy-lipids employed (Fig. 1F).

163 To conclude, the use of fusogenic liposomes enabled rapid incorporation of approximately 4  
164  $\times 10^6$  specific lipids into the PM per living cell over 10 minutes. Based on our tracking data,  
165 we estimated that each cell contains around 300 caveolae, comprising around 0.1% of the  
166 surface area. Caveolae are approximately 60 nm in diameter, and each lipid occupies 0.62 Å.  
167 Therefore *ca.*  $10 \times 10^6$  lipids are contained within the caveolae, of which 50% is Chol. The  
168 amount of specific incorporated lipids in our system is therefore about half of the total  
169 amount of lipids contained within caveolae. The immediate addition of extra lipids to the PM  
170 did not result in a detectable effect on the cell volume (Fig. S1H).

#### 171 **GSLs and Chol decrease the surface stability of caveolae**

172 We next aimed to elucidate if lipids are involved in controlling the balance between stable  
173 and dynamic caveolae at the PM and if effects could be attributed to individual lipid species.  
174 To visualize caveolae, we generated a stable mammalian Flp-In T-Rex HeLa cell line  
175 expressing Cav1-mCherry, hereafter named Cav1-mCh HeLa cells. Protein expression was  
176 induced by doxycycline (Dox) to achieve expression of Cav1-mCherry at similar level as  
177 endogenous Cav1 (Fig. S1I). Using TIRF microscopy and single-particle tracking, we  
178 determined the time each Cav1-mCh positive punctuate structure spent at the PM (track  
179 duration) and the speed of an object (track mean speed) in, or close to, the PM (see Method  
180 section for detailed tracking parameters). Given the previously reported surface dynamics of  
181 caveolae (Boucrot et al., 2011; Mohan et al., 2015; Pelkmans & Zerial, 2005), we postulated  
182 that stable caveolae will have a long duration and low speed, limited by their lateral diffusion  
183 in the PM (Fig. 2A, “Stable”). Caveolae that scission off (“Scissioned intermediate”) or re-  
184 fuse (“Fused intermediate”) with the PM during the recording period will have an

185 intermediate duration and moderate speed, whereas caveolae that undergo rounds of scission  
186 and fusion (“Surface adjacent”), but remain close to the surface will have a high speed and  
187 short duration as they are not stably fused with the PM and short duration (Fig. 2A). We did  
188 indeed observe a clear correlation between the track duration and track mean speed where, in  
189 general, short tracks exhibited higher speeds, whereas long tracks displayed lower speeds  
190 (Figs. 2B and S2A). Although the numbers of caveolae in each cell were similar at the  
191 beginning and end of the recording, we found that the number of tracks far exceeded the  
192 caveolae numbers (Fig. S2C). This was expected as surface adjacent caveolae would give rise  
193 to several tracks. However, a drop in the fluorescent signal just below the set threshold value,  
194 would also contribute to a divided track resulting in an overestimation of short tracks versus  
195 long tracks. Therefore, we did not consider the average durations as absolute, but rather used  
196 them to compare between experimental runs with differing conditions. To verify that the  
197 tracking was sensitive to differences in caveolae dynamics, we depleted cells of EHD2,  
198 which has been shown to stabilize caveolae to the cell surface (Morén et al., 2012; Stoeber et  
199 al., 2012). Particle tracking analysis showed that the pool of surface adjacent caveolae  
200 increased, while the pool of stable caveolae decreased (Figs. 2B’ and S2A’). When the  
201 average track duration was considered, this translated into a 0.65 fold change compared to  
202 control cells (Fig. 3E), proving that the particle tracking indeed was sensitive enough to  
203 register caveolae dynamic changes.

204 Next, we screened the influence of different lipid species on caveolae mobility at the PM  
205 using tracking. To do this, fusogenic liposomes loaded with relevant lipids were added to  
206 Cav1-mCh HeLa cells and TIRF movies were recorded immediately (Fig. 2C). PE was used  
207 in control liposomes as it is abundant in the PM. Following incorporation of PE, caveolae  
208 dynamics remained unchanged, showing that the fusion of liposomes did not obstruct  
209 caveolae dynamics (Figs. 2D and S2B). In comparison to controls, Bodipy-labeled GSLs

210 (GM1 and LacCer) and Chol significantly reduced the lifetime of caveolae at the cell surface  
211 as indicated by decreased track duration measurements (Figs. 2C-D and S2B, Video 1-3).  
212 Besides enhanced mobility, caveolae also showed an increase in mean speed (Fig. 2E). For  
213 example, the treatment with LacCer gave a ratio of surface adjacent caveolae versus stable  
214 that was comparable to the EHD2 depletion (Figs. 2B', S2A' and S2F-G). A direct  
215 comparison between LacCer and Cer revealed that Cer did not enhance caveolae dynamics in  
216 a similar fashion (Figs. 2D and S2B). No difference in the number of caveolae present in the  
217 PM was observed before and after the addition of liposomes (Fig. S2D). To verify that the  
218 effect was not due to the the Bodipy label, we treated cells with liposomes containing either  
219 unlabeled LacCer [(LacCer(d18:1/16:0))] or unlabeled Chol and quantified the track duration.  
220 This showed that the unlabeled lipids had the same effect on the track duration as the  
221 corresponding Bodipy-labeled analogues (Fig. 2F).

222 When cells were treated with Bodipy-SM C<sub>12</sub>, most of the caveolae were stable at the PM  
223 (Fig. 2C-D). This was characterized by a dramatic increase in track duration, and a reduction  
224 of the track mean speed (Figs. 2D and 2E). To further investigate the role of SM, we  
225 analyzed caveolae duration following SMase treatment, and found that this resulted in a  
226 decreased track duration, in agreement with a surface-stabilizing role for this lipid (Fig. 2G).

### 227 **Chol and GSLs induce surface release of caveolae via an EHD2-dependent mechanism**

228 EHD2 normally localizes with the majority of surface associated caveolae (Morén et al.,  
229 2012). We aimed to address if the increased caveolae dynamics induced by either Chol or  
230 GSLs were due to their PM release, as characterized by loss of the stabilizing protein EHD2.  
231 Therefore, we treated Cav1-mCh HeLa cells with the different fusogenic liposomes and  
232 visualized endogenous EHD2 using indirect immunofluorescent labeling (Fig. 3A). These  
233 experiments revealed that incorporation of GM1, LacCer or Chol into the PM led to a

234 significantly lower amount of EHD2 localized to Cav1 (Fig. 3A, scatter plot). This data  
235 suggests that the caveolae release induced by increased PM levels of LacCer and Chol is due  
236 to a loss of EHD2-mediated stabilization. Conversely, Cer and SM C<sub>12</sub>, as well as its short  
237 chain analogue SM C<sub>5</sub>, did not appear to have any significant effect on the association of  
238 EHD2 with Cav1 (Fig. 3A, scatter plot). Further experiments showed that, following lipid  
239 treatment, the majority of caveolae remained associated with cavin1, revealing that no  
240 disruption of the caveolae coat, and subsequent release of cavin1, occurred (Fig. 3B).

241 Since increased scission of caveolae from the cell surface results in more mobile intracellular  
242 caveolar vesicles (Stoeber et al., 2012), we performed fluorescence recovery after  
243 photobleaching (FRAP) experiments to investigate the recovery rate of caveolae. Addition of  
244 LacCer resulted in more mobile caveolae inside the cells in comparison with control cells  
245 (Figs. 3C-D). The recovery rate after LacCer addition was similar to the rate in EHD2-  
246 depleted cells (Figs. 3C-D). This further supported the hypothesis that lipid incorporation  
247 drives caveolae scission (Figs. 3C-D). When LacCer or Chol was added to EHD2-depleted  
248 cells we found that the caveolae track duration was not further reduced in comparison with  
249 EHD2-depleted cells (Figs. 3E and S3A-B). These results supported our hypothesis that the  
250 lipid-induced effect on caveolae dynamics was due to the loss of surface stability mediated  
251 by EHD2, and implied that EHD2 controls the stability of caveolae in response to lipid  
252 composition. To test whether increased levels of EHD2 could restore caveolae stability after  
253 lipid treatment, we transiently expressed BFP-tagged EHD2 in Cav1-mCh HeLa cells.  
254 Analysis of TIRF live cell movies showed that EHD2-BFP positive caveolae were highly  
255 stable compared to controls (Fig. 3F). In the presence of EHD2-BFP, the destabilizing effect  
256 seen for GM1, LacCer and Chol was abolished, as demonstrated by negligible changes in  
257 track duration compared to control conditions (Fig. 3F). This suggested that excess levels of  
258 EHD2 were capable of restricting the effect of excess Chol and GSLs. In addition, tracking of

259 caveolae in cells stably expressing Cav1-mCh and EHD2-BFP showed that most of the  
260 caveolae were positive for EHD-BFP (96%) and that this population was more stable than the  
261 population lacking EHD2-BFP (4%) (Figs. S3C-E). Surprisingly, SM C<sub>12</sub> had an additive  
262 effect and led to predominantly stable caveolae (Fig. 3F), implying that the increased cell  
263 surface stability of caveolae may be due to changes in membrane fluidity rather than EHD2  
264 recruitment.

265 To test if the suppression of the lipid effect by increased levels of EHD2 relied on multiple  
266 rounds of assembly and disassembly of EHD2 at caveolae, we overexpressed a BFP-tagged  
267 ATP-cycle mutant, EHD2-I157Q. The increased ATP hydrolysis rate of this mutant leads to  
268 stable association of EHD2-I157Q to caveolae and a slower exchange rate (Fig. S3F)  
269 (Daumke et al., 2007; Hoernke et al., 2017; Stoeber et al., 2012). We observed that, when co-  
270 expressed in Cav1-mCh cells, both EHD2-I157Q and EHD2 stabilized caveolae at the PM to  
271 similar extents, independent of treatment with either LacCer or Chol (Fig. S3G). This verified  
272 that stable assembly, but not disassembly of EHD2, is necessary to stabilize caveolae.

273 To clarify whether, in order to have a stabilizing role, EHD2 had to be caveolae-associated  
274 prior to lipid addition, fluorescently labeled, purified EHD2 (EHD2-647) was microinjected  
275 into Cav1-mCh HeLa cells (Fig. 3G). Within 20 min, EHD2-647 colocalized with Cav1,  
276 confirming that the microinjected protein was indeed recruited to caveolae (Figs. S3H-I).  
277 Next, we tested if an acute injection of EHD2-647 could rescue the effect on caveolae  
278 dynamics caused by LacCer. Strikingly, we found that exogenously added EHD2 stabilized  
279 the caveolae to the same extent as the overexpressed EHD2, demonstrating that increased  
280 levels of EHD2 can acutely reverse the increased mobility of caveolae induced by lipids (Fig.  
281 3H).

282 **LacCer and Chol accumulate in caveolae and Chol is sequestered within these domains**

283 As GSLs and Chol increased the surface release of caveolae, we aimed to determine whether  
284 there was a differential accumulation of lipids within caveolae at the PM. We treated Cav1-  
285 mCh HeLa cells with fusogenic liposomes and followed the distribution of Bodipy-labeled  
286 LacCer or Chol using live-cell TIRF microscopy. After 15 min, both lipids were found to  
287 colocalize with Cav1-mCh positive structures (Figs. 4A and S4A, Video 5-6). Data analysis  
288 was hindered by high caveolae mobility following lipid addition, and the extent of  
289 colocalization could not be quantified. To circumvent this, we overexpressed EHD2-BFP to  
290 stabilize caveolae at the PM. Interestingly, nearly 80% of caveolae positive for EHD2 were  
291 also positive for LacCer or Chol (Figs. 4B-B' and S4B, Video 7-8). In comparison, Cer,  
292 which had no effect on caveolae dynamics, did not localize to caveolae, even in the presence  
293 of EHD2-BFP (Figs. S4C-D).

294 To investigate the exchange of lipids between the stable caveolae and the surrounding PM,  
295 we performed FRAP experiments. The Bodipy-LacCer signal reappeared rapidly at precisely  
296 the bleached spot positive for Cav1-mCh, with a close to quantitative fluorescence recovery  
297 (Figs. 4C-D). This indicated that the lipid diffused freely throughout the PM and, following  
298 photobleaching, re-accumulated quickly within caveolae. In comparison, Bodipy-Chol  
299 recovered much slower with 60% of the initial signal being restored after 5 min acquisition  
300 time (Figs. 4D and S4E). This showed that there is a large immobile pool of Chol in caveolae  
301 that is sequestered from the rest of the PM. Our data suggests that both LacCer and Chol are  
302 highly enriched in caveolae and, while the lateral diffusion of LacCer in and out of caveolae  
303 is high, Chol is restrained to this invagination.

#### 304 **Chol accumulation reduces the caveolae diameter in 3T3-L1 adipocytes**

305 To elucidate whether lipid accumulation affected the overall morphology of surface  
306 connected caveolae in Cav1-mCh HeLa cells, we analyzed their ultrastructure in cells

307 overexpressing EHD2. Since the number of caveolae in the PM of these cells is relatively  
308 low, we used correlative light electron microscopy (CLEM) to specifically identify  
309 fluorescently tagged caveolae by combining light microscopy with the higher resolution  
310 images of transmission electron microscopy (TEM). Fluorescence light microscopy images of  
311 Cav1-mCh HeLa cells were superimposed with correlative electron micrographs to find the  
312 closest match of fluorescence signal to structure using the nuclear stain as a guide (Figs. 5A  
313 and S5A). The surface connected caveolae in cells treated with LacCer and Chol, displayed a  
314 similar flask-shaped morphology as seen in control cells, verifying that lipid addition did not  
315 majorly distort caveolae morphology. To be able to quantitatively assess differences in  
316 morphology, we differentiated 3T3-L1 cells to adipocytes, which results in upregulation of  
317 Cav1 and EHD2 (Fig. S5B) (Morén et al., 2019), and formation of a large number of  
318 caveolae (Thorn et al., 2003) that could be clearly distinguished from clathrin-coated pits  
319 (Fig. S5C). These cells also provided a more physiologically relevant system since  
320 adipocytes are the main source of cholesterol storage and efflux (Krause & Hartman, 1984).  
321 Quantification of lipid incorporation verified that fusogenic liposomes could be used to insert  
322 specific lipids into the PM of these cells (Fig. S5D). Using TEM, we analyzed the dimensions  
323 of caveolae before and after Chol addition (Figs. 5B-E). We found that the neck diameter of  
324 surface associated caveolae were significantly decreased and more homogeneous following  
325 Chol incorporation in comparison to control cells (Fig. 5D). Furthermore, the bulb width was  
326 also significantly smaller resulting in more drop-shaped caveolae (Fig. 5D'). Quantitative  
327 analysis of the spherical population of caveolae without surface connected necks allowed us  
328 to measure the surface area of caveolae. Comparison to control cells showed that area, as well  
329 as bulb width, decreased following Chol addition (Figs. 5E-E'). Furthermore, Chol  
330 incorporation resulted in a more homogeneous caveolae population in terms of size and  
331 dimensions. These data suggested that an acute increase in Chol levels in the PM of 3T3-

332 adipocytes induced alterations in the caveolae coat architecture resulting in reduced neck  
333 diameter and a smaller more uniform bulb diameter.

334 **GSLs are internalized to the endosomal system independent of Cav1, while Chol is**  
335 **predominantly trafficked to lipid droplets**

336 Next, we aimed to address if caveolae scission significantly contributed to internalization and  
337 trafficking of lipids in our system as previously proposed (Le Lay et al., 2006; Puri et al.,  
338 2001; Shvets et al., 2015). We used fusogenic liposomes to investigate if Bodipy-labeled  
339 LacCer or Chol were internalized and trafficked through the endosomal pathway following  
340 incorporation into the PM. To mark early endosomes (EE), Rab5-BFP was transiently  
341 expressed in Cav1-mCh HeLa cells. Cells were incubated with fusogenic liposomes for either  
342 15 min or 3 h, followed by fixation and EE localization was quantified. We observed  
343 localization of LacCer to the EE but not to the Golgi, contrasting previous studies using  
344 BSA-Bodipy-LacCer (Puri et al., 2001). After 15 min, more than half of the EE were positive  
345 for LacCer (55%) compared to only 6% for Chol (Figs. 6A-B). After 3 h, the number of  
346 LacCer-positive EE remained constant, whereas the EE positive for Chol had increased to  
347 18% (Fig. 6B). To test if caveolae were involved in lipid trafficking to the EE, the  
348 experiments were repeated in cells depleted of Cav1 (Figs. 6C-E). After 15 min incubation  
349 time, 55% and 10% of EE were positive for LacCer and Chol, respectively (Figs. 6C-D). This  
350 suggested that while caveolae did not seem to influence the efficiency of LacCer or Chol  
351 trafficking to endosomes, loss of Cav1 resulted in an increased amount of Chol accumulating  
352 in this compartment. Our data indicate that caveolae serve as buffers or sensors of GSL and  
353 Chol concentrations rather than endocytic vesicles.

354 During our experiments, we noticed that a large fraction of Chol localized to compartments  
355 distinct from the endosomal system. To determine whether Chol localized to lipid droplets  
356 (LD) as previously proposed (Le Lay et al., 2006; Shvets et al., 2015), we incubated HeLa



357 cells with fusogenic liposomes and visualized LD using LipidTOX-DR. On average, 85% of  
358 LDs were positive for Chol after both 15 min and 3 h (Figs. 6F-G), and similar levels of  
359 Chol-positive LD were detected in cells lacking Cav1 (Fig. 6H). While Chol extensively  
360 localized to LD, we did not observe LacCer associated with LD (Figs. 6F-G). These data are  
361 consistent with the hypothesis that excess Chol in the PM is trafficked directly to LD in a  
362 process that does not require caveolae per se, and that the levels of Chol taking an alternative  
363 route to EE increases in the absence of caveolae.

## 364 **DISCUSSION**

365 While PM turnover is typically regulated in a tightly controlled manner, marginal changes in  
366 its composition are associated with severe diseases such as cancer, diabetes, and Alzheimer's  
367 disease (Harayama & Riezman, 2018). It has been proposed that caveolae play a major role in  
368 preserving lipid homeostasis via sensing and buffering PM properties (Parton & del Pozo,  
369 2013; Pilch & Liu, 2011). However, studies detailing how lipid composition influences  
370 cellular phenotypes have been hindered by a lack of methods to selectively manipulate the  
371 PM lipid composition; especially with regard to introducing specific lipids. To address this,  
372 we applied an approach for studying these systems in living cells that employs  
373 DOPE/DOTAP-based liposomes capable of mediating highly effective fusion processes with  
374 cell membranes to deliver their lipid cargoes. Such liposomes have previously been used as  
375 nanocarriers to deliver intracellular proteins (Kube et al., 2017). Our methodology  
376 successfully delivered specific lipids into the PM bilayer of living cells with high efficiency.  
377 These rapid fusion events enabled us to study, for the first time, how caveolae respond to an  
378 acute change in PM lipid composition and to observe lipid exchange in the caveolae bulb.  
379 Furthermore, the use of labelled lipids allowed us to measure the levels of incorporation in  
380 relation to endogenous levels. Our results demonstrate the power of this approach for

381 studying caveolae dynamics and we foresee that our methodology will also be a useful tool  
382 outside of this framework.

383 Our work shows that the surface association of caveolae is highly sensitive to changes in the  
384 PM lipid composition. An acute increase in the levels of Chol and GSLs, which were found  
385 to specifically accumulate in caveolae, dramatically increased caveolae mobility. These  
386 caveolae traveled at higher speeds, their PM duration was shorter and they also displayed  
387 reduced levels of EHD2, a protein indicative of PM-associated caveolae. Therefore, we  
388 conclude that accumulation of Chol and GSLs in caveolae trigger surface release of caveolae.  
389 In agreement with this, analysis by EM revealed that the caveolae neck diameter was reduced  
390 in cells with elevated Chol levels. Our findings are consistent with previous reports  
391 suggesting that BSA-LacCer and Chol decrease the number of caveolae associated to the PM  
392 and enhance their mobility (Le Lay et al., 2006; Sharma et al., 2004). Based on the present  
393 study, increased caveolae mobility is a direct result of lipid accumulation in these structures.  
394 As our methodology allowed us to determine the levels of specifically incorporated lipids, we  
395 found that rapid, yet relatively small increases in specific lipids can affect caveolae dynamics.  
396 Because caveolae immediately responded to these changes in bilayer composition, we  
397 propose that they serve as PM sensors, not only for membrane tension, but also for lipid  
398 composition.

399 Previous studies have suggested that a threshold concentration of Chol is required to maintain  
400 caveolae integrity and proposes that assembly and disassembly is in a dynamic equilibrium  
401 dependent on Chol levels (Hailstones et al., 1998). This is also in line with our experiments  
402 showing that excess Chol drives caveolae assembly towards scission and that Chol was  
403 indeed found to accumulate in caveolae when these structures were restrained to the surface  
404 by EHD2 overexpression. Furthermore, our methodology enabled, for the first time,

405 measurement of lipid lateral flow in and out of the caveolae bulb using FRAP. Comparing the  
406 FRAP recovery of Bodipy-LacCer and Bodipy-Chol, which were both enriched in caveolae,  
407 showed that while photobleached Bodipy-LacCer was almost fully exchanged via lateral  
408 diffusion after 2 min, photobleached Bodipy-Chol was only exchanged by 50%. This showed  
409 that Chol was sequestered in caveolae, potentially through its interaction to Cav1 (Parton &  
410 del Pozo, 2013). In contrast, Bodipy-Cer, which lacks the disaccharide structural motif of  
411 LacCer, did not accumulate in caveolae and had no effect on their dynamics, which is in  
412 agreement with earlier reports (Sharma et al., 2004). Precisely how the lactosyl group  
413 mediates the caveolae-enrichment of LacCer and how this in turn drives caveolae scission is  
414 not clear. Interestingly, we found that Bodipy-SM C<sub>12</sub>, but not Bodipy-SM C<sub>5</sub>, dramatically  
415 increased caveolae stability, in terms of both speed and duration. The contrasting effects of  
416 SM analogues were highly intriguing as both lipids are thought to partition into a liquid  
417 disordered phase in artificial giant unilamellar vesicles (Klymchenko & Kreder, 2014).  
418 However, the altered chain length might influence their interactions with Chol. Interestingly,  
419 SM has been shown to sequester a pool of Chol in the PM, which, together with an accessible  
420 and inaccessible pool, aid in the sensing of the Chol levels in the PM (Das et al., 2014). The  
421 elevated levels of SM C<sub>12</sub> may alter the levels of SM-sequestered Chol, thereby affecting  
422 caveolae stability. In agreement with this, we found that a dramatic reduction of SM using  
423 SMase indeed increased the dynamics of caveolae.

424 While an area of extensive research, a consensus on the exact mechanism of caveolae  
425 scission has not yet been reached. Our observations suggest a model, where the accumulation  
426 of lipids in caveolae reduces the neck diameter, leading to scission. We speculate that this  
427 could be due to increased access of scission-mediating molecules like dynamin to the neck, or  
428 that that these lipids promote assembly of Cav1 and cavins, which drive curvature towards  
429 scission. The lipid-driven assembly of Cav1 may be an intrinsically unstable system,

430 eventually resulting in scission if no restraining forces are applied. This indicates that  
431 scission is tightly coupled to, and a continuum of caveolae biogenesis. In line with this,  
432 expression of caveolin in bacterial systems induced the formation of internal caveolae-like  
433 vesicles containing caveolin so-called heterologous caveolae (Walser et al., 2012). The  
434 scission step could also involve lipid phase separation. A similar mechanism has previously  
435 been proposed but not experimentally validated (Lenz et al., 2009), and our new data shows  
436 that a locally increased concentration of GSLs and Chol in caveolae may induce phase  
437 separation and therefore facilitate budding and scission of caveolae. Consistent with this,  
438 model membrane studies have shown that sterol-induced phase separation can promote  
439 membrane scission (Bacia et al., 2005; Roux et al., 2005). Of interest, in other systems GSLs  
440 and Chol have been suggested to play a crucial role in membrane nanodomain budding to  
441 generate intracellular transport carriers (Schuck & Simons, 2004).

442 EHD2 has been shown to confine caveolae to the cell surface (Morén et al., 2012; Stoeber et  
443 al., 2012). In the current study, we acutely altered the lipid composition in order to induce  
444 caveolae scission and analyzed the immediate role of EHD2. We found that removal of  
445 EHD2, while at the same time changing the lipid composition, did not have an additive effect  
446 on caveolae dynamics. However, excess levels of EHD2 due to overexpression or direct  
447 microinjection, could suppress the effect of the altered lipid composition. This suggests that  
448 an increased assembly rate of EHD2 at the caveolae neck is necessary and sufficient to drive  
449 the equilibrium towards stable surface association of caveolae. We conclude that oligomers  
450 of EHD2 might provide a restraining force that prevents reduction of the neck diameter and  
451 thereby inhibits phase separation or assembly of scission-mediating proteins. Similarly,  
452 EHD2 would prevent flattening of caveolae and thus, act to stabilize the typical bulb-shape of  
453 caveolae. Therefore, EHD2 would act as a key regulator of caveolae dynamics in response to  
454 changes in both PM lipid composition and membrane tension.

455 Caveolae have been proposed to play an integral role in intracellular lipid trafficking (Le Lay  
456 et al., 2006; Puri et al., 2001; Shvets et al., 2015). This prompted us to examine the cellular  
457 fate of our labelled lipids. We found that while Bodipy-LacCer was internalized via the  
458 endosomal system, Chol predominately localized to LD. Importantly, and in contrast to  
459 previous data (Shvets et al., 2015), we found that loss of caveolae did not majorly influence  
460 the trafficking of these lipids in HeLa cells. Based on this, we propose that caveolae should  
461 not be considered as vehicles for internalization of lipids, but rather that lipid composition  
462 influences caveolae biogenesis and dynamics. Together with the caveolae coat components, it  
463 is feasible that sequestered lipids may control formation and define the size and curvature of  
464 these PM invaginations. This, together with our data showing that Chol is enriched and  
465 sequestered in caveolae, implies that caveolae could serve as reservoirs of Chol in the PM,  
466 thereby buffering the surface levels of this lipid.

467 Together, our findings indicate that the dynamic behavior of caveolae is highly sensitive to  
468 changes in PM lipid composition. We demonstrate that, following incorporation into the lipid  
469 bilayer, GSLs and Chol accumulate in caveolae, which promotes scission of these membrane  
470 invaginations from the cell surface. The current study redefines the fundamental  
471 understanding of how caveolae dynamics are governed by biologically relevant lipids and  
472 will be of future relevance linking caveolae malfunction with lipid disorders.

## 473 MATERIALS AND METHODS

### 474 Reagents.

475 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-3-  
476 trimethylammonium-propane (chloride salt) (DOTAP), TopFluor<sup>®</sup>-cholesterol (Bodipy-  
477 Chol), TopFluor<sup>®</sup>- phosphatidylethanolamine (Bodipy-PE), D-lactosyl- $\beta$ -1,1' N-palmitoyl-D-  
478 erythro-sphingosine [LacCer(d18:1/16:0)] and Lyso-Lactosylceramide (Lyso-LacCer) were  
479 purchased from Avanti Polar Lipids Inc. (Alabaster, AL, US). Bodipy<sup>™</sup> FL C5-ganglioside  
480 GM1 (Bodipy-GM1), Bodipy<sup>™</sup> FL C5-ceramide (Bodipy-Cer), Bodipy<sup>™</sup> FL C<sub>12</sub>-  
481 spinghomyelin (Bodipy-SM C<sub>12</sub>), Bodipy<sup>™</sup> FL C<sub>5</sub>-spinghomyelin (Bodipy-SM C<sub>5</sub>) were  
482 obtained from Thermo Fisher Scientific (Waltham, MA, US). BODIPY<sup>™</sup> FL-C5 NHS ester  
483 (4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid, succinimidyl  
484 ester) was purchased from Setareh Biotech, LLC (Eugene, OR, US). Cholesterol (Chol), d7-  
485 cholesterol, *N,N*-diisopropylethylamine, sphingomyelinase (SMase) from *Bacillus cereus*,  
486 myriocin from *Mycelia sterilia*, anhydrous dimethylformamide (DMF), chloroform (CHCl<sub>3</sub>)  
487 and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO, US). LC-MS  
488 grade formic acid was purchased from VWR Chemicals (Radnor, PA, US). LC-MS grade 2-  
489 propanol and acetonitrile from Merck Millipore (Billerica, MA, US). Milli-Q<sup>®</sup> water (Merck  
490 Millipore) was used. All reagents and chemicals were used without further purification.

### 491 Bodipy-LacCer synthesis.

492 Thin layer chromatography was performed on aluminum backed silica gel plates (median  
493 pore size 60 Å, fluorescent indicator 254 nm, Fisher Scientific, Hampton, NH, US) and  
494 visualized by exposure to UV light (365 nm) and stained with acidic ethanolic vanillin  
495 solution. Flash chromatography was performed using chromatography grade silica gel  
496 (0.035-0.070 mm, 60Å, Thermo Fisher Scientific). NMR spectra were recorded on a Bruker

497 AVANCE (600 MHz) spectrometer. <sup>1</sup>H Chemical shifts are reported in  $\delta$  values relative to  
498 tetramethylsilane and referenced to the residual solvent peak (CD<sub>3</sub>OD:  $\delta_{\text{H}} = 3.31$  ppm,  $\delta_{\text{C}} =$   
499 49.00 ppm). Coupling constants are reported in Hz.

500 Lyso-LacCer (5 mg, 8  $\mu\text{M}$ ) was dissolved in DMF (200  $\mu\text{l}$ ) and *N,N*-diisopropylethylamine  
501 (2.1  $\mu\text{l}$ , 12  $\mu\text{M}$ , 1.5 eq.) was added. BODIPY<sup>TM</sup> Fl-C5 NHS ester (66  $\mu\text{l}$  of a stock solution of  
502 5 mg/100  $\mu\text{l}$  DMF, 8  $\mu\text{M}$ , 1.0 eq.) was added and the reaction was shielded from light and  
503 stirred for 14 h. The reaction mixture was concentrated and purified by column  
504 chromatography (CHCl<sub>3</sub>, MeOH, H<sub>2</sub>O, 70:15:2 – 65:25:2) to afford the product Bodipy-  
505 LacCer (6.5 mg, 88%, Fig. S1A) as a red film (Gretskaya & Bezuglov, 2013).

506 Retention factor:  $R_{\text{f}} = 0.46$  (CHCl<sub>3</sub>, MeOH, H<sub>2</sub>O, 65:25:2)

507 NMR data: <sup>1</sup>H-NMR (CD<sub>3</sub>OD, 600 MHz)  $\delta$  7.41 (1H, s), 7.03 (1H, d,  $J=4.1$  Hz), 6.36 (1H, d,  
508  $J=4.0$  Hz), 6.18 (1H, s), 5.67 (1H, dt,  $J=15.3, 6.8$  Hz), 5.44 (1H, dd,  $J=15.3, 7.7$  Hz), 4.34  
509 (1H, d,  $J=7.7$  Hz), 4.29 (1H, d,  $J=7.8$  Hz), 4.17 (1H, dd,  $J=10.1, 4.7$  Hz), 4.07 (1H, t,  $J=7.9$   
510 Hz), 3.99 (1H, ddd,  $J=8.2, 4.6, 3.3$  Hz), 3.89 (1H, dd,  $J=12.1, 2.6$  Hz), 3.84 (1H, dd,  $J=12.2,$   
511 4.3 Hz), 3.81 (1H, d,  $J=3.1$  Hz), 3.78 (1H, dd,  $J=11.4, 7.5$  Hz), 3.70 (1H, dd,  $J=11.5, 4.6$  Hz),  
512 3.61 – 3.59 (1H, m), 3.62 – 3.51 (3H, m), 3.52 – 3.49 (1H, m), 3.47 (1H, dd,  $J=9.7, 3.3$  Hz),  
513 3.39 (1H, ddd,  $J=9.3, 4.0, 2.7$  Hz), 3.28 (1H, t,  $J=8.5$  Hz), 2.94 (2H, t,  $J=7.3$  Hz), 2.50 (3H,  
514 s), 2.28 (3H, s), 2.25 (2H, t,  $J=7.0$  Hz), 2.00 – 1.93 (2H, m), 1.79 – 1.66 (4H, m), 1.37 – 1.20  
515 (31H, m, (11-CH<sub>2</sub>)), 0.89 (3H, t,  $J=7.0$  Hz).

516 <sup>13</sup>C-NMR (CD<sub>3</sub>OD, 151 MHz)  $\delta$  175.7, 160.9, 160.2, 145.0, 136.1, 135.2, 134.9, 131.2,  
517 130.0, 125.6, 120.9, 117.9, 105.1, 104.5, 80.6, 77.1, 76.5, 76.3, 74.8, 74.8, 73.0, 72.5, 70.3,  
518 69.9, 62.5, 61.8, 54.8, 37.1, 33.4, 33.1, 30.8, 30.8, 30.8, 30.8, 30.8, 30.7, 30.5, 30.4, 30.3,  
519 29.5, 29.4, 27.0, 23.7, 14.9, 14.5, 11.2.

520 **Cell lines and primary cultures.**

521 HeLa cells (ATCC-CRM-CCL-2) were cultured in Dulbecco's Modified Eagle Medium  
522 (DMEM, Thermo Fisher Scientific) supplemented with 10% (v/v) Fetal bovine serum (FBS,  
523 Thermo Fisher Scientific) at 37°C, 5% CO<sub>2</sub>. For generation of HeLa Flp-In T-REx  
524 Caveolin1-mCherry cells the pcDNA/FRT/TO/Caveolin1-mCherry construct was generated  
525 by exchanging the EGFP-tag in the pcDNA/FRT/TO/Caveolin1-EGFP (Mohan et al., 2015)  
526 for a mCherry tag by restriction cloning using enzymes AgeI and NotI (Thermo Fisher  
527 Scientific). The HeLa Flp-In T-REx EHD2-BFP-P2A-Caveolin1-mCherry construct was  
528 generated by linearizing pcDNA/FRT/TO/Caveolin1-mCh with the restriction enzyme  
529 HindIII (Thermo Fisher Scientific). The DNA encoding EHD2-BFP and the P2A peptide was  
530 inserted by Gibson assembly using NEBuilder HiFi DNA assembly master mix (New  
531 England BioLabs, Ipswich, MA, USA). The Flp-In TRex HeLa cell lines were maintained in  
532 DMEM supplemented with 10% (v/v) FBS, 100 µg/ml hygromycin B (Thermo Fisher  
533 Scientific), and 5 µg/ml blasticidin S HCl (Thermo Fisher Scientific) for plasmid selection at  
534 37°C, 5% CO<sub>2</sub>. Expression at endogenous levels was induced by incubation with 0.5 ng/ml  
535 (Cav1-mCh) and 1.0 ng/ml (EHD2-BFP-P2A-Cav1mCh) doxycycline hyclate (Dox, Sigma-  
536 Aldrich) for 16-24 h.

537 3T3-L1 fibroblasts (ATC-CL-173) were maintained in DMEM supplemented with 10% (v/v)  
538 FBS and penicillin-streptomycin (10000 U/ml, 1:100, Thermo Fisher Scientific) at 37°C, 5%  
539 CO<sub>2</sub> and differentiated to adipocytes as previously describe (Zebisch et al., 2012). Briefly,  
540 cells were either seeded directly into a 6-well plate or on glass coverslips in a 6-well plate at  
541  $6 \times 10^5$  cells/well (day -3 of differentiation). The cells reached confluency the following day  
542 and the medium was changed (day -2). After 48 h (day 0) the medium was exchanged for  
543 differentiation medium I [supplemented DMEM containing 0.5 mM 3-isobutyl-1-



544 methylxanthine (IBMX, Sigma Aldrich), 0.25  $\mu$ M dexamethasone (Dex, Sigma Aldrich), 1  
545  $\mu$ g/ml insulin (Sigma Aldrich) and 2  $\mu$ M rosiglitazone (Cayman Chemical, Ann Arbor, MI,  
546 USA)]. Following incubation for 48 h, the medium was changed to differentiation medium II  
547 (supplemented DMEM containing 1  $\mu$ g/ml insulin) (day 2). Experiments were performed on  
548 day 4 of differentiation.

#### 549 **Fusogenic liposomes.**

550 Liposomes were prepared from a lipid mixture of DOPE, DOTAP and either Bodipy-tagged  
551 lipid or unlabeled lipid at a ratio of 47.5:47.5:5. Lipid blends were in MeOH:CHCl<sub>3</sub> (1:3,  
552 v/v). Following the generation of a thin film using a stream of nitrogen gas, the vesicles were  
553 formed by addition of 20 mM HEPES (VWR, Stockholm, SE, pH 7.5, final lipid  
554 concentration 2.8  $\mu$ mol/ml) and incubated for 1.5 h at room temperature. Glass beads were  
555 added to facilitate rehydration. The liposome dispersion was sonicated for 30 min  
556 (Transsonic T 310, Elma, Singen, DE). The hydrodynamic diameters (*z*-average) of the  
557 liposomes were measured using dynamic light scattering with a Malvern Zetasizer Nano-S  
558 (Malvern Instruments, Worcestershire, UK). Samples were diluted 1:100 in 20 mM HEPES  
559 (pH 7.5) and measured using a UV-transparent disposable cuvettes (Sarstedt, Nümbrecht,  
560 DE). The measurements were performed at 20°C. The Nano DTS Software 5.0 was used for  
561 acquisition and analysis of the data.

#### 562 **Lipid quantification by LC-ESI-MS/MS.**

563 One day prior to experiment, cells were seeded in a 6-well plate. Cells were left untreated or  
564 treated with 11.7 nmol/ml of the different fusogenic liposomes for 10 min at 37°C, 5% CO<sub>2</sub>.  
565 The cells were washed three times with PBS and harvested in 500  $\mu$ l MeOH by scraping.  
566 Counting revealed that approximately  $4 \times 10^5$  cells were obtained per sample. For myriocin

567 (2.5  $\mu\text{M}$ ) and SMase (0.01 U) treatment, cells were incubated for 24 h or 2 h, respectively.  
568 Extraction was performed using a mixer mill set to a frequency 30 Hz for 2 min, with 1  
569 tungsten carbide bead added to each tube. Thereafter the samples were centrifuged at 4°C,  
570 14000 RPM, for 10 min. A volume of 260  $\mu\text{l}$  of the supernatant was transferred to micro vials  
571 and evaporated under  $\text{N}_2$  (g) to dryness. The dried extracts were stored at -80°C until  
572 analysis. Calibration curves of Bodipy-labeled standards (Bodipy-SM  $\text{C}_{12}$  and Bodipy-  
573 LacCer) as well as standards for endogenous LacCer and SM [LacCer(d18:1/16:0) and  
574 SM(d18:1/16:0)] were prepared prior to analysis. Stock solutions of each compound were  
575 prepared at a concentration of 500 ng/ $\mu\text{l}$  and stored at -20°C. A 5-point calibration curve  
576 (0.025-0.4 ng/ $\mu\text{l}$ ) was prepared by serial dilutions [Bodipy-SM  $\text{C}_{12}$   $R^2 = 0.9909$ ;  
577 LacCer(d18:1/16:0)  $R^2 = 0.9945$ ; Bodipy-LacCer  $R^2 = 0.9983$ ; LacCer(d18:1/14:0)  $R^2 =$   
578 0.8742], except for endogenous SM(d18:1/16:0) where 0.025-10.0 ng/ $\mu\text{l}$  was used ( $R^2 =$   
579 0.9991). Samples and calibration curves were analyzed using a 1290 Infinity system from  
580 Agilent Technologies (Waldbronn, Germany), consisting of a G4220A binary pump, G1316C  
581 thermostated column compartment and G4226A autosampler with G1330B autosampler  
582 thermostat coupled to an Agilent 6490 triple quadrupole mass spectrometer equipped with a  
583 jet stream electrospray ion source operating in positive ion mode. Separation was achieved  
584 injecting 2  $\mu\text{l}$  of each sample (resuspended in 20  $\mu\text{l}$  of MeOH) onto a CSH  $\text{C}_{18}$  2.1x50 mm,  
585 1.7  $\mu\text{m}$  column (Waters, Milford, MA, USA) held at 60°C in a column oven. The gradient  
586 eluents used were 60:40 acetonitrile:H<sub>2</sub>O (A) and 89:10.5:0.4 isopropanol:acetonitrile:water  
587 (B), both with 10 mM ammonium formate and 0.1% formic acid, with a flow rate of 500  
588  $\mu\text{l}/\text{min}$ . The initial conditions consisted of 15% B, and the following gradient was used with  
589 linear increments: 0-1.2 min (15-30% B), 1.2-1.5 (30-55% B), 1.5-4.0 (55% B), 4.0-4.8 (55-  
590 100% B), 4.8-6.8 (100% B), 7.1-8.0 (15% B). The MS parameters were optimized for each

591 compound (Table 1). The fragmentor voltage was set at 380 V, the cell accelerator voltage at  
592 5 V and the collision energies from 20-30 V, nitrogen was used as collision gas.

593 Jet-stream gas temperature was at 150°C with a gas flow of 16 l/min. The sheath gas  
594 temperature was kept at 350°C with a gas flow of 11 l/min. The nebulizer pressure was set to  
595 35 psi and the capillary voltage was set at 4 kV. The QqQ was run in Dynamic MRM Mode  
596 with using a retention time delta of 0.8 min and 500 millisecond cycle scans. The data was  
597 quantified using custom scripts (Swedish Metabolomics Centre, Umeå, Sweden).

598 **Table 1.** Retention times (RT), MRM-transition stages monitored (precursor ion and product  
599 ions) and collision energies of analyzed compounds.

600

Compounds	MRM transition		RT (min)	Collision Energy (V)
	Precursor Ion	Product Ion		
Bodipy-LacCer	926.5	562.4	1.48	30
LacCer(d18:1/16:0)	862.6	520.5	2.84	20
LacCer(d18:1/14:0)	834.6	264.3	2.8	40
SM(d18:1/16:0)	703.6	184.1	2.9	30
Bodipy-SM C <sub>12</sub>	865.6	184.1	2.12	30

## 601 **Cholesterol quantification by GC-MS**

602 One day prior to experiment cells were seeded in a 6-well plate. Cells were left untreated or  
603 treated with 11.7 nmol/ml fusogenic liposomes for 10 min at 37°C, 5% CO<sub>2</sub>. The cells were  
604 washed three times with PBS and harvested in 250 µl MeOH by scraping and two wells were  
605 pooled to generate approximately  $8 \times 10^5$  cells per 500 µl sample into Eppendorf tubes.  
606 Extraction was performed using a mixer mill set to a frequency 30 Hz for 2 min, with 1  
607 tungsten carbide bead added to each tube. Obtained extracts were centrifuged at 4°C, 14000  
608 RPM for 10 min. A volume of 300 µl of the collected supernatants were transferred to

609 individual micro vials and the extracts were dried under N<sub>2</sub> (g) to dryness. Separate  
610 calibration curves were prepared for endogenous and d7-Chol. A 6-point calibration curve  
611 spanning from 0-10 ng/μl was prepared for d7-Chol (R<sup>2</sup> = 0.9909). For endogenous Chol a 6-  
612 point calibration curve spanning from 0-500 ng/μl was prepared (R<sup>2</sup> = 0.9969). Methyl  
613 stearate at a final concentration of 5ng/μl was used as internal standard in both calibration  
614 curves. Derivatization was performed according to a previously published method (Gullberg  
615 et al., 2004) . In detail, 10 μl of methoxyamine (15 μg/μl in pyridine) was added to the dry  
616 sample that was shaken vigorously for 10 min before left to react in room temperature. After  
617 16 hours 10 μl of MSTFA was added, the sample was shaken and left to react for 1 hour in  
618 room temperature. A volume of 10 μl of methyl stearate (15 ng/μl in heptane) was added  
619 before analysis. For d7-cholesterol quantification, 1 μl of the derivatized sample was injected  
620 by an Agilent 7693 autosampler, in splitless mode into an Agilent 7890A gas chromatograph  
621 equipped with a multimode inlet (MMI) and 10 m x 0.18 mm fused silica capillary column  
622 with a chemically bonded 0.18 μm DB 5-MS UI stationary phase (J&W Scientific). The  
623 injector temperature was 250°C. The carrier gas flow rate through the column was 1 ml min<sup>-1</sup>,  
624 the column temperature was held at 60°C for 1 min, then increased by 60°C min<sup>-1</sup> to 300°C  
625 and held there for 2 min. The column effluent is introduced into the electron impact (EI) ion  
626 source of an Agilent 7000C QQQ mass spectrometer. The thermal AUX 2 (transfer line) and  
627 the ion source temperatures were 250°C and 230°C, respectively. Ions were generated by a  
628 70 eV electron beam at an emission current of 35 μA and analyzed in dMRM-mode. The  
629 solvent delay was set to 3 min. For a list of MRM transitions see Table 2. For endogenous  
630 Chol analysis, the samples were reanalyzed in split mode (10:1) together with the Chol  
631 calibration curve. Data were processed using MassHunter Qualitative Analysis (Agilent  
632 Technologies, Atlanta, GA, USA) and custom scripts (Swedish Metabolomics Centre, Umeå,  
633 Sweden).

634 **Table 2.** MRM transitions for labeled and endogenous Chol.

635

Compound	Comment	Precursor ion	MS1 resolution	Product ion	MS2 resolution	RT	RT delta Min (total)
<b>Methyl stearate</b>	IS-std	298	Unit	101.1	Unit	5.6	2
<b>Chol</b>	Quant	329	Unit	95	Unit	7.8	2
<b>Chol</b>	Qual	368	Unit	213	Unit	7.8	2
<b>d7-Chol</b>	Quant	336	Unit	95	Unit	7.8	2
<b>d7- Chol</b>	Qual	375	Unit	213	Unit	7.8	2

636

637 **Calculations of the number of PM lipids.**

638 The average PM area of fibroblast is around 3000  $\mu\text{m}^2$  (Sheetz et al., 2006), of which 23% is  
639 estimated to be occupied by proteins (Dupuy & Engelman, 2008), which translates into that  
640 the average PM of a cell contains approximately  $7 \times 10^9$  lipids (Alberts et al., 2002). Our data  
641 is in agreement with these reported values, whereby our measured values for SM(d18:1/16:0)  
642 being 40% of total SM species (Kjellberg et al., 2014), and 21mol% of PM lipids translating  
643 to  $9.6 \times 10^9$  lipids in the PM.

644 **Assessment of lipid incorporation into the PM with live-cell spinning disk microscopy.**

645 One day prior to the experiment, non-induced Cav1-mCh HeLa cells or 3T3-L1 adipocytes  
646 were seeded on glass coverslips (CS-25R17 or CS-25R15, Warner Instruments, Hamden, CT,  
647 US) in a 6-well plate at  $3 \times 10^5$  cells/well (37°C, 5% CO<sub>2</sub>). Live cell experiments were  
648 conducted in phenol red-free DMEM (live cell medium, Thermo Fisher Scientific)  
649 supplemented with 10% FBS and 1 mM sodium pyruvate (Thermo Fisher Scientific) at 37°C  
650 in 5% CO<sub>2</sub>. To follow the distribution of Bodipy throughout the PM, a POC mini 2 chamber  
651 (PeCon, Erbach, DE) was used that allowed addition of the fusogenic liposomes during data  
652 acquisition. Liposomes were added at a concentration of 7 nmol/ml and movies of confocal  
653 stacks were recorded every 30 s over a period of 5 min using a 63X lens and Zeiss Spinning

654 Disk Confocal controlled by ZEN interface with an Axio Observer.Z1 inverted microscope,  
655 equipped with a CSU-X1A 5000 Spinning Disk Unit and an EMCCD camera iXon Ultra  
656 from ANDOR. For TIRF movies the same system was used but employing a 100X lens and  
657 an Axio Observer.Z1 inverted microscope equipped with an EMCCD camera iXonUltra from  
658 ANDOR. The increase in fluorescence intensity (FI) of the Bodipy signal was measured  
659 within circular regions of interest, which were either evenly distributed over the PM seen in  
660 the confocal section or over the basal PM in the case of TIRF. The total FI was determined by  
661 calculating integrated density (area x FI), which was then background corrected. Ten regions  
662 of interest (ROIs) per cell were analyzed using Zeiss Zen interface ( $n = 3$ , two independent  
663 experiments). Based on that lipids occupy  $65 \text{ \AA}^2$ , which translates to  $3.1 \times 10^6$  lipid  
664 molecules/ $\mu\text{m}^2$  (Dopico, 2007), and that the mean liposomes diameter was 225 nm,  
665 corresponding to an area of  $0.19 \mu\text{m}^2$ , we calculated that each liposome contained  $0.6 \times 10^6$   
666 lipids, of which 5% were Bodipy-labeled. To estimate the cell volume, the cell surface was  
667 segmented with the surface feature within the Imaris x64 9.1.2 (Bitplane, Zurich, CH) using  
668 the mCherry fluorescence.

### 669 **Constructs, transfections and cell treatments.**

670 pTagBFP-C (Evrogen, Moscow, RU) was used to generate the expression constructs of Rab5  
671 and EHD2 wt or I157Q. Cav1-mCh HeLa cells were transfected with Lipofectamine<sup>TM</sup> 2000  
672 (Thermo Fisher Scientific) using Opti-MEM<sup>TM</sup> I reduced serum medium (Thermo Fisher  
673 Scientific) for transient protein expression. For EHD2 and Cav1 depletion, Cav1-mCh HeLa  
674 cells were transfected with either stealth siRNA, specific against human EHD2 or human  
675 Cav1, or scrambled control (all from Thermo Fisher Scientific) using Lipofectamine<sup>TM</sup> 2000  
676 and Opti-MEM according to manufacturer's instructions unless otherwise stated. Cells were  
677 transfected twice over a period of 72 h before the experiment. Protein levels were analyzed

678 by SDS-PAGE and immunoblotting using rabbit anti-EHD2 (Morén et al., 2012) and rabbit  
679 anti-Cav1 antibodies (Abcam, Cambridge, UK). Mouse anti-clathrin heavy chain (clone 23,  
680 BD Transduction Laboratories, San Jose, CA, US) was used as loading control. Cells were  
681 treated with 2.5  $\mu$ M myriocin in complete medium 24 h prior to harvesting. SMase was  
682 added to cells to generate a final concentration of 0.01 units in complete medium 2 h prior to  
683 harvesting or live cell imaging.

#### 684 **Analysis of caveolae dynamics.**

685 To track caveolae dynamics, induced Cav1-mCh HeLa cells were treated with fusogenic  
686 liposomes (7 nmol/ml) and 5 min TIRF movies were recorded with an acquisition time of 3 s.  
687 Imaris software was used for tracking analysis of Cav1-mCh positive structures, which were  
688 segmented as spots and structures with a diameter of 0.4  $\mu$ m were selected. The applied  
689 algorithm was based on Brownian motion with max distance travelled of 0.8  $\mu$ m and a max  
690 gap size of 4. Experiments where EHD2 (wt and mutant) was either transiently expressed or  
691 depleted were performed and analyzed the same way. Colocalization of EHD2 (wt and  
692 mutant) to Cav1-mCh was quantified with Imaris software. Within a ROI, spots were created  
693 in one channel (*e.g.*, red channel) and the second channel (*e.g.*, blue channel) was masked.  
694 The masked spots show only colocalized red and blue spots and the percentage was  
695 correlated to the original channel. The analysis of the dynamic behavior of caveolae positive  
696 for or lacking EHD2-BFP was performed using double Flp-In EHD2-BFP Cav1-mCh HeLa  
697 cells. The tracking was done as described above and the data from the tracks of Cav1-mCh  
698 spots lacking EHD2-BFP was collected and removed from the data of Cav1-mCh spots  
699 positive for EHD2-BFP. Statistical analysis was performed on track duration (s) and track  
700 mean speed ( $\mu$ m/s) data and data is shown as fold change. All micrographs and acquired  
701 movies were prepared with Fiji (Schindelin et al., 2012) and Adobe Photoshop CS6.

702 **Intracellular trafficking of lipids.**

703 Induced Cav1-mCh HeLa cells were seeded on glass coverslips (CS-25R15) in a 6-well plate  
704 at  $3 \times 10^5$  cells/well (37°C, 5% CO<sub>2</sub>). On the following day, the cells were incubated with  
705 fusogenic liposomes (7 nmol/ml) for 15 min or 3 h. Rab5-BFP (Francis et al., 2015) was  
706 either transiently expressed or. To analyze the localization of lipids to lipid droplets (LDs),  
707 induced Cav1-mCh HeLa cells were treated with lipids for 15 min or 3 h, fixed and stained  
708 with HCS LipidTOX™ Deep Red Neutral Lipid Stain (1:200, Thermo Fisher Scientific).  
709 Confocal stacks were acquired on Zeiss Spinning Disk Confocal microscope. The  
710 colocalization was analyzed as described above. Micrographs were prepared with Fiji  
711 (Schindelin et al., 2012) and Adobe Photoshop CS6.

712 **Immunostaining.**

713 Induced Cav1-mCh HeLa cells were seeded on precision coverslips (No. 1.5H, Paul  
714 Marienfeld GmbH & Co. KG, Lauda-Königshofen, DE) in 24-well plates at  $50 \times 10^3$   
715 cells/well and incubated overnight (37°C, 5% CO<sub>2</sub>). Following incubation with fusogenic  
716 liposomes (7 nmol/ml) for 1 h, the cells were washed thrice with phosphate-buffered saline  
717 (PBS, pH 7.4). Cells were fixed with 4 % PFA in PBS (Electron Microscopy Sciences,  
718 Hatfield, PA, US) and subsequent permeabilization and blocking was carried out  
719 simultaneously using PBS containing 5% goat serum and 0.05% saponin. Cells were then  
720 immunostained with rabbit anti-EHD2 (Morén et al., 2012) and rabbit anti-PTRF (Abcam)  
721 followed by goat anti-rabbit IgG secondary antibody coupled to Alexa Fluor 647 (Thermo  
722 Fisher Scientific) as previously described (Lundmark et al., 2008). Confocal images were  
723 acquired using the Zeiss Spinning Disk Confocal microscope (63X lens). Pearson  
724 colocalization coefficients were obtained using Imaris software applying the Coloc feature  
725 with automatic thresholding. All Pearson coefficients were derived from two independent



726 experiments for the EHD2 stain. Analysis of the colocalization of cavin 1 and Cav1-mCh was  
727 repeated once. Data from at least 30 images was analyzed with images containing 2–3 cells  
728 on average. Micrographs were prepared using Fiji (Schindelin et al., 2012) and Adobe  
729 Photoshop CS6.

### 730 **FRAP experiments.**

731 Induced Cav1-mCh HeLa cells were seeded on glass coverslips (CS-25R15) in a 6-well plate  
732 at  $3 \times 10^5$  cells/well and incubated overnight (37°C, 5% CO<sub>2</sub>). Cells were treated with 7  
733 nmol/ml of Bodipy-labeled liposomes for 10 min followed by two washes with live cell  
734 media before imaging using TIRF using a Zeiss Axio Observer.Z1 inverted microscope.  
735 Three reference images were recorded before a ROI was photobleached for 1000 ms using  
736 maximal laser intensity (488 nm or 561 nm). The fluorescent recovery images were taken  
737 every 3 s for 5 min. For the lipid incorporation experiment, a region within the PM with  
738 homogeneous fluorescence was chosen. FRAP of the EHD2 mutants was performed the same  
739 way. For the LacCer and Chol accumulated in caveolae, FRAP was performed between 15 to  
740 60 min after lipid addition and regions with structures positive for Cav1-mCh, EHD2-BFP  
741 and Bodipy-lipid were selected. For FRAP experiments that quantified the recovery of Cav1-  
742 mCh, induced Cav1-mCh HeLa cells were either untreated, depleted of EHD2 using siRNA  
743 or incubated with Bodipy-LacCer liposomes. FRAP experiments were performed as  
744 described above using the Zeiss Spinning Disk Confocal microscope (63X lens). The signal  
745 recovery monitored in focal plane close to the basal membrane. The intensities of the  
746 bleached regions were corrected for background signal and photobleaching of the cell. Data  
747 from at least 10 cells were collected per condition and mean FRAP recovery curves were  
748 plotted using Prism 5.0 (GraphPad, San Diego, CA, US).

### 749 **Microinjection.**

750 Mouse EHD2 cysteine mutant construct (L303C,C96S, C138S, C356S) was expressed as N-  
751 terminal His<sub>6</sub>-tag fusion proteins in *Escherichia coli* Rosetta (DE3) and purified (Daumke et  
752 al., 2007). Dithiothreitol was removed from the protein using PD-10 columns and the protein  
753 was labelled with Alexa Fluor™ 647 C2 Maleimide (Thermo Fisher Scientific) (Hoernke et  
754 al., 2017). The protein was diluted to a concentration of 0.5 mg/ml in 150 mM NaCl, 20 mM  
755 HEPES pH 7.5 and 1 mM MgCl<sub>2</sub>. Cav1-mCh HeLa cells were transfected with siRNA and  
756 induced as described above. One day prior to the injection experiment, Cav1-mCh HeLa cells  
757 were seeded in MatTek dishes (35 mm dish, high tolerance 1.5, MatTek Corporation,  
758 Ashland, MA, US) with a cell density of  $3 \times 10^5$  cells/dish and induced with Dox. In the case  
759 of LacCer addition, the cells were treated with 7 nmol/ml of Bodipy-LacCer fusogenic  
760 liposomes for 10 min followed by two washes with live cell media before microinjection.  
761 Microinjection was performed with Injectman NI2 coupled to the programmable  
762 microinjector Femtojet (Eppendorf, Hamburg, DE). The protein was loaded in Femtotips II  
763 (Eppendorf) and injection was done with an injection pressure of 1.0 hPa, compensation  
764 pressure of 0.5 hPa and injection time of 0.1 s. Live images were acquired on TIRF every 3 s  
765 for a total of five min using a Nikon Eclipse Ti-E inverted microscope with a 100X lens  
766 (Apochromat 1.49 Oil 0.13-0.20 DIC N2, Nikon). Z-stacks of injected cells were captured  
767 using a 60X lens (Apochromat 1.40 Oil DIC, Nikon). Tracking of Cav1-mCh and  
768 colocalization analysis was done with Imaris as previously described.

#### 769 **Correlative light electron microscopy.**

770 Cav1-mCh cells transiently expressing EHD2-GFP alone or treated with either Bodipy-  
771 LacCer or Bodipy-Chol liposomes were fixed in 2% paraformaldehyde (PFA) and 0.2% of  
772 glutaraldehyde (Taab Laboratory Equipment Ltd, Aldermaston, UK) in 0.1 M phosphate  
773 buffer (pH 7.4) for 1-2 h and then stored in 1% PFA at 4°C. For the grid preparation, the cells

774 were scraped into the fixative solution and washed thrice with PBS (pH 7.4) and once with  
775 PBS containing 0.1% glycine (pH 7.4, Merck Millipore, Burlington, US). The cell pellet was  
776 embedded in 12% gelatin (Dr. Oetker, food grade) in 0.1 M phosphate buffer (pH 7.4).  
777 Blocks of around 1 mm<sup>2</sup> were cut and cryo-protected by overnight infiltration in 2.3 M  
778 sucrose (VWR) in 0.1 M phosphate buffer. Next, the blocks were plunge frozen in liquid  
779 nitrogen. The sample block was sectioned at -120°C to obtain 80 nm sections. These were  
780 mounted in a drop of in 0.1 M phosphate buffer containing 1:1 of 2% methyl cellulose  
781 (Sigma-Aldrich) and 2.3 M sucrose on TEM grids with a carbon-coated Formvar film (Taab  
782 Laboratory Equipment Ltd). The grids were incubated with PBS (pH 7.4) at 37°C for 20 min  
783 and stained with DAPI (4',6-Diamidino-2-Phenylindole, Dilactate, 1:1000 in PBS, pH 7.4,  
784 Thermo Fisher Scientific) before imaging on a Nikon Eclipse Ti-E inverted microscope with  
785 a 100X lens (Apochromat 1.49 Oil 0.13-0.20 DIC N2, Nikon). Low magnification images  
786 were taken at 20X for orientation on the grid and to aid the overlay of fluorescent microscopy  
787 images and with the higher resolution images of TEM. Contrasting for TEM was done by  
788 embedding the grids in 1.8% methyl cellulose and 0.4% uranyl acetate (Polysciences, Inc.,  
789 Hirschberg an der Bergstrasse, DE) solution prepared in water (pH 4) for 10 min in the dark.  
790 TEM was performed with a Talos 120C transmission electron microscope (FEI, Eindhoven,  
791 NL) operating at 120kV. Micrographs were acquired with a Ceta 16M CCD camera (FEI)  
792 using Maps 3.3 (FEI, Hillsboro, OR, US). The fluorescent images were overlaid atop TEM  
793 images of the same cells collected from the ultrathin section using Adobe Photoshop CS6.

#### 794 **Electron microscopy.**

795 3T3-L1 cells were seeded on MatTek dishes (35 mm dish, high tolerance 1.5) and  
796 differentiated to adipocytes as described above. 3T3-L1 adipocytes were untreated or  
797 incubated with Bodipy-Chol liposomes for 45 min, washed with PBS and fixed as follows.

798 All chemical fixation steps were performed using a microwave (Biowave, TED PELLA, inc.)  
799 unless stated and solutions were prepared and rinses were performed in 0.1M cacodylate  
800 buffer (Sigma-Aldrich) or water. Fixation of the cells was performed in 0.05% malachite  
801 green oxalate (Sigma-Aldrich) and 2.5% gluteraldehyde (Taab Laboratory Equipment Ltd,  
802 Aldermaston, UK) in cacodylate buffer. The samples were rinsed four times with cacodylate  
803 buffer and post-fixed with 0.8%  $K_3Fe(CN)_6$  (Sigma-Aldrich) and 1%  $OsO_4$  (Sigma-Aldrich)  
804 in cacodylate buffer and rinsed four times with cacodylate buffer. The samples were then  
805 stained with 1% aqueous tannic acid (Sigma-Aldrich). Following two rinses in cacodylate  
806 buffer and water, samples were stained with 1% aqueous uranyl acetate (Polysciences, Inc.,  
807 Hirschberg an der Bergstrasse, DE). After four washed with water, samples were dehydrated  
808 in gradients of ethanol (25%, 50%, 75%, 90%, 95%, 100% and 100%) (VWR). The samples  
809 were infiltrated with graded series of hard grade spurr resin (Taab Laboratory Equipment Ltd,  
810 Aldermaston, UK) in ethanol (1:3, 1:1 and 3:1) and then left in 100% resin for 1 h at room  
811 temperature. The samples were later polymerized overnight at 60°, sectioned and imaged  
812 with a Talos 120C transmission electron microscope (FEI, Eindhoven, NL) operating at  
813 120kV. To obtain quantitative data, segmentation of caveolae for measurement of bulb width  
814 and measurement of neck diameter for surface-connected caveolae was performed with “icy”  
815 (de Chaumont et al., 2012). In order to extract bulb width and surface area, “active cells”  
816 plug-in was used with three points to make an elliptical contour that fitted individual  
817 caveolae. The neck diameter was obtained by drawing a ROI across the neck of surface-  
818 connected caveolae. The analysis was performed blinded and with randomized sections.

819 **Statistical analysis.**

820 Statistical analysis was carried out by two-tailed unpaired Student *t*-test for comparison with  
821 control samples using GraphPad Prism 5.0 software. All experiments were performed at least  
822 twice with data representing mean  $\pm$  SEM unless otherwise stated.

## 823 **ONLINE SUPPLEMENTAL MATERIAL**

824 Fig. S1 illustrates dynamic behavior of caveolae at the cell surface and shows  
825 characterization of liposomes as well as chromatography data. Fig. S2 shows the correlation  
826 between track duration and track mean speed and the effect of GSLs and Chol on lifetime of  
827 caveolae. Fig. S3 shows the colocalization of EHD2 to Cav1 in double Flp-In cells, analysis  
828 of track duration in presence of EHD2-I157Q-BFP and TIRF images of TIRF images of  
829 Cav1-mCh HeLa cells with or without microinjection of EHD2-647. Fig. S4 shows that  
830 Bodipy-labeled LacCer and Chol accumulate in caveolae at the PM and demonstrates the  
831 recovery of lipids within caveolae after photobleaching. Fig. S5 shows CLEM approach and  
832 upregulation of EHD2 and Cav1 in 3T3-L1 adipocytes. Video 1-4 show cell surface  
833 dynamics of Cav1-mCh untreated (Video 1) or following addition of Bodipy-LacCer (Video  
834 2), Chol (Video 3) or Bodipy-SM C<sub>12</sub> (Video 4). Video 5-6 show colocalization of Bodipy-  
835 LacCer (Video 5) or Bodipy-Chol (Video 6) with Cav1-mCh positive structures. Video 7-8  
836 show accumulation of Bodipy-LacCer (Video 7) or Chol (Video 8) in Cav1-mCh positive  
837 structures in presence of EHD2-BFP.

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#### 847 **AUTHOR CONTRIBUTIONS**

848 M.H., E.L. and R.L. designed the research; M.H., E.L., N.G.V.G. and R.L. performed  
849 research and analyzed data; L.W.K.M. synthesized lipids, M.A. and A.J. performed mass  
850 spectrometry, M.H., E.L., L.W.K.M. and R.L. wrote the paper.

#### 851 **DECLARATION OF INTERESTS**

852 The authors declare no competing interests.

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1055

1056 **FIGURE LEGENDS**

1057 **Fig. 1. Rapid insertion of Bodipy-labeled lipids into the PM of living cells using**  
1058 **fusogenic liposomes.**

1059 **(A)** Fusogenic liposomes are used to insert Bodipy-labeled lipids into the PM. Their rapid  
1060 distribution is followed in real time using TIRFM. **(B)** Image sequence of Bodipy-LacCer  
1061 distribution throughout basal membrane of HeLa cells. Total Bodipy fluorescence intensity  
1062 (FI) was measured within ROIs (yellow insert) using Zeiss Zen interface.  $n = 10$ , three  
1063 independent experiments, mean  $\pm$  SEM. **(C)** Quantification of endogenous SM(d18:1/16:0)  
1064 using LC-ESI-MS/MS in untreated control cells or cells treated with SMase or myriocin for 2  
1065 h or 24 h, respectively. Data are shown as mean + SD. **(D)** Quantification of Bodipy- or d7-  
1066 labeled lipids (black bars) and endogenous lipids (grey bars) in cells following incubation of  
1067 cells with fusogenic liposomes. Analysis was performed using mass spectrometry. Data are  
1068 shown as mean + SD. **(E)** Incorporation rate of Bodipy-lipids into PM of live cells. HeLa  
1069 cells were treated with fusogenic liposomes (final total lipid concentration 7 nmol/mL). Total  
1070 Bodipy fluorescence intensity (FI) was measured within circular ROIs (see insert) in a  
1071 confocal section using spinning disk microscopy. Ten ROIs were analyzed using the Zeiss  
1072 Zen system software.  $n \geq 2$ , two independent experiments, mean  $\pm$  SEM. Scale bars, 10  $\mu$ m.  
1073 **(F)** TIRF FRAP of Bodipy-lipids after incorporation into PM of HeLa cells. A circular ROI  
1074 was photobleached and recovery of Bodipy FI was monitored over 5 min. Bodipy FI was  
1075 normalized to background and reference.  $n \geq 10$ , mean  $\pm$  SEM.

1076 **Fig. 2. GSLs and Chol decrease the surface stability of caveolae.**

1077 **(A)** Scheme showing different dynamic behaviors of caveolae. **(B, B')** Distribution of track  
1078 mean speed amongst subpopulations of track duration of Cav1-mCh structures **(B)** and after  
1079 EHD2 depletion **(B')**. Five datasets for each condition were analyzed from TIRF live cell  
1080 movies. **(C)** Representative images from TIRF movies of Cav1-mCh HeLa cells and after 15  
1081 min incubation with liposomes containing Bodipy-lipids. Color-coded trajectories illustrate  
1082 time that structures can be tracked at PM over 5 min (dotted square). Scale bars, 10  $\mu$ m. See  
1083 Video 1-4. **(D-E)** Quantification of track duration of Cav1-mCh structures from TIRF movies  
1084 after incubation with liposomes containing labeled **(D)** or unlabeled lipids **(E)**. Fold changes  
1085 are relative to control (Cav1-mCh). **(D)**  $n \geq 8$ , at least two independent experiments; **(E)**  $n \geq$   
1086 8, two independent experiments, \*\*\*,  $P \leq 0.001$  vs. control. **(F)** Quantification of track mean  
1087 speed of Cav1-mCh structures from TIRF movies (same cells as in D). **(G)** Quantification of  
1088 track duration of Cav1-mCh structures from TIRF movies following incubation with SMase  
1089 for 2 h. Fold changes are relative to control (Cav1-mCh).  $n \geq 5$ . All analyses were performed  
1090 using Imaris software and data are shown as mean + SEM.

1091 **Fig. 3. Chol and GSLs induce surface release of caveolae via an EHD2-dependent**  
1092 **mechanism.**

1093 **(A)** Representative images of maximum projected confocal z-stacks of Cav1-mCh HeLa  
1094 cells. Untreated cells or cells treated with LacCer-Bodipy liposomes for 1 h, fixed and  
1095 immunostained for endogenous EHD2. High-magnification images (dotted square) show  
1096 localization of EHD2 to Cav1-mCh (see scatterplot for quantification).  $n \geq 60$ , two  
1097 independent experiments, mean  $\pm$  SEM. \*\*\*,  $P \leq 0.001$  vs. control. **(B)** Experimental  
1098 protocols analogous to (A), with exception of endogenous cavin1 immunostaining.  $n \geq 60$ ,  
1099 mean  $\pm$  SEM. **(C)** Confocal FRAP of Cav1-mCh HeLa cells treated with either EHD2 siRNA  
1100 or Bodipy-LacCer liposomes. A ROI was photobleached and recovery of mCherry FI  
1101 monitored over 5 min. mCherry FI was normalized to background and reference.  $n \geq 10$ ,  
1102 mean  $\pm$  SEM. **(D)** Representative time-lapse series showing control Cav1-mCh HeLa cells  
1103 and cells treated with either EHD2 siRNA or Bodipy-LacCer liposomes. The photobleached  
1104 area is outlined with white circles. mCherry FI is intensity-coded using LUT. **(E)** Effects of  
1105 lipids on track duration of Cav1-mCh structures were analyzed following siRNA-mediated  
1106 depletion of EHD2.  $n \geq 8$ , two independent experiments, mean + SEM. **(F)** Quantification of  
1107 track duration of Cav1-mCh HeLa cells transiently expressing EHD2-BFP with or without  
1108 incubation with liposomes. Changes in track duration are relative to control (indicated by  
1109 dotted line).  $n \geq 8$ , two independent experiments, mean + SEM. \*\*\*,  $P \leq 0.001$  vs. control  
1110 cells. **(G)** Representative live cell confocal image of EHD2-647 microinjected into Cav1-  
1111 mCh HeLa cells. **(H)** Quantification of track duration of Cav1-mCh cells treated with  
1112 Bodipy-LacCer and following microinjection of EHD2-647.  $n = 8$ , mean + SEM. All scale  
1113 bars, 10  $\mu\text{m}$ .

1114 **Fig. 4. LacCer and Chol accumulate in caveolae and Chol is sequestered within these**  
1115 **domains.**

1116 **(A, B)** Cav1-mCh HeLa cells **(A)** and Cav1-mCh HeLa cells transiently expressing EHD2-  
1117 BFP **(B)** were incubated with Bodipy-LacCer liposomes. White lines indicate location of  
1118 kymograph and the corresponding intensity profiles illustrate localization of Bodipy-LacCer  
1119 to Cav1-mCh either alone or in presence of EHD2-BFP. Intensity profiles are relative to  
1120 maximum values for each sample. Scale bars, 10  $\mu\text{m}$ ; kymograph scale bars, 5  $\mu\text{m}$ . See Video  
1121 S3 and S4. **(B')** Quantification of EHD2-positive caveolae colocalizing with lipids.  $n \geq 8$ , at  
1122 least two independent experiments, mean + SEM. **(C)** Cav1-mCh HeLa cells transiently  
1123 expressing EHD2-BFP were incubated with Bodipy-lipids for 10 min. Following  
1124 photobleaching, recovery of Bodipy signal within caveolae was monitored over time. White  
1125 arrows highlight surface connected caveolae with accumulated Bodipy-LacCer. Scale bar, 5  
1126  $\mu\text{m}$ . **(D)** Recovery curves of Bodipy intensities within bleached membrane ROI. Bodipy FI  
1127 was normalized to background and reference.  $n \geq 10$ , mean  $\pm$  SEM.

1128 **Fig. 5. Chol accumulation reduces the caveolae diameter in 3T3 adipocytes**

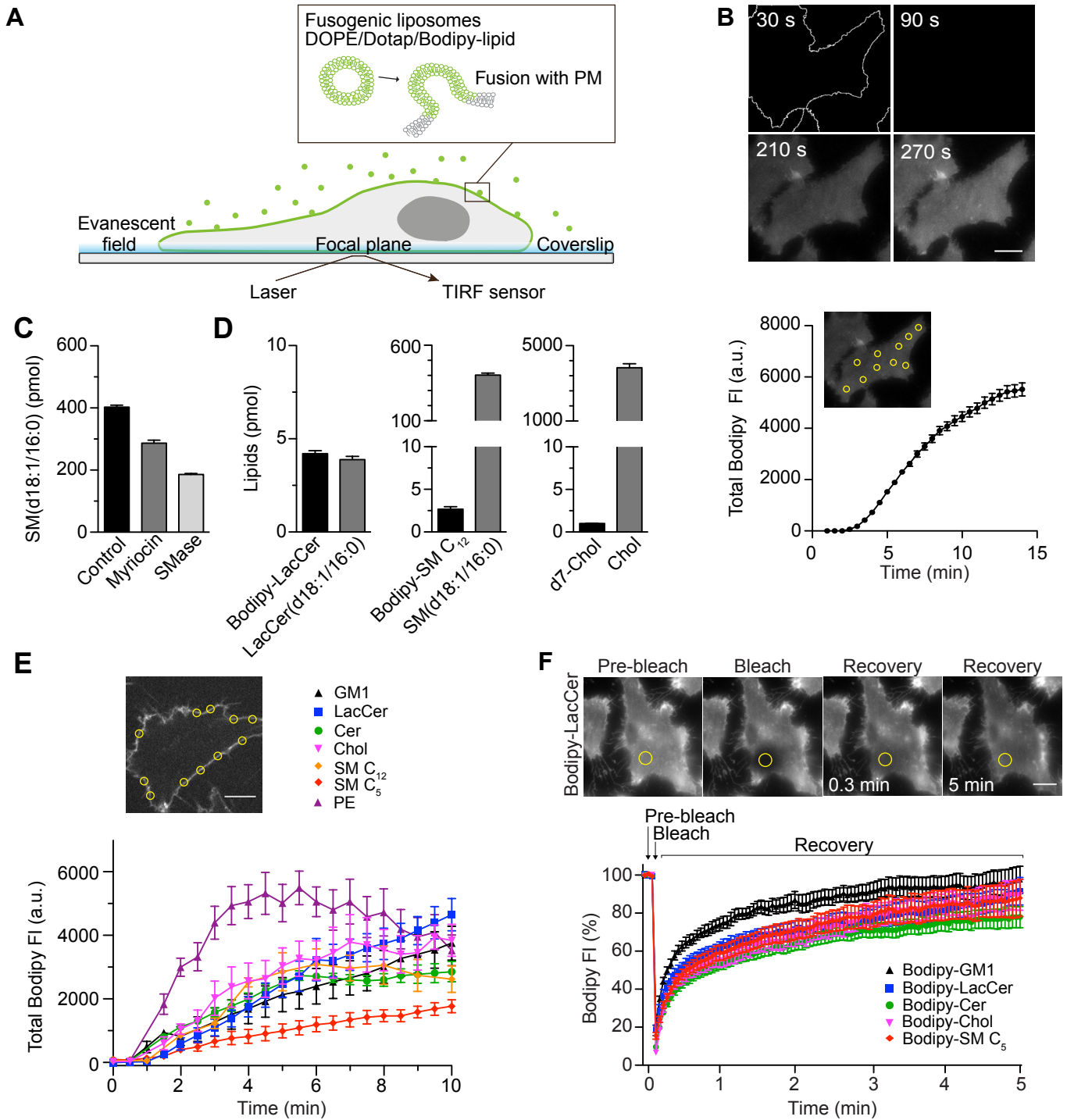
1129 **(A)** Representative overlays of light microscopy images with corresponding electron  
1130 micrographs showing localization of caveolae (Cav1-mCh in red) and nuclei (DAPI in cyan)  
1131 for untreated Cav1-mCh HeLa cells or cells treated with Bodipy-labeled Chol or LacCer.  
1132 Dotted boxes show regions of higher magnification in corresponding panels below. N,  
1133 nucleus; PM, plasma membrane. White arrows denote surface connected caveolae and black  
1134 arrows indicate surface adjacent caveolae. Scale bars, 1  $\mu\text{m}$ ; inset scale bars, 100 nm. **(B, C)**  
1135 Representative electron micrographs of control 3T3-L1 adipocytes **(B)** and 3T3-L1  
1136 adipocytes treated with Bodipy-Chol **(C)**. Cells were chemically fixed, embedded in resin  
1137 and processed for electron microscopy. Scale bars, 100 nm. **(D, D')** Scatter plots showing the  
1138 quantification of neck diameter **(D)** and bulb width **(D')** of surface connected caveolae in  
1139 3T3-L1 adipocytes. Bulb width and neck diameter are highlighted in (B), upper panel.  $n \geq 30$ ,  
1140 mean  $\pm$  SEM. **(E, E')** Scatter plots showing the quantification of surface area **(E)** and bulb  
1141 width **(E')** of surface adjacent caveolae in 3T3-L1 adipocytes.  $n \geq 120$ , mean  $\pm$  SEM. \*\*\*,  $P$   
1142  $\leq 0.001$ .

1143 **Fig. 6. GSLs are internalized to the endosomal system independent of Cav1, while Chol**  
1144 **is predominantly trafficked to lipid droplets.**

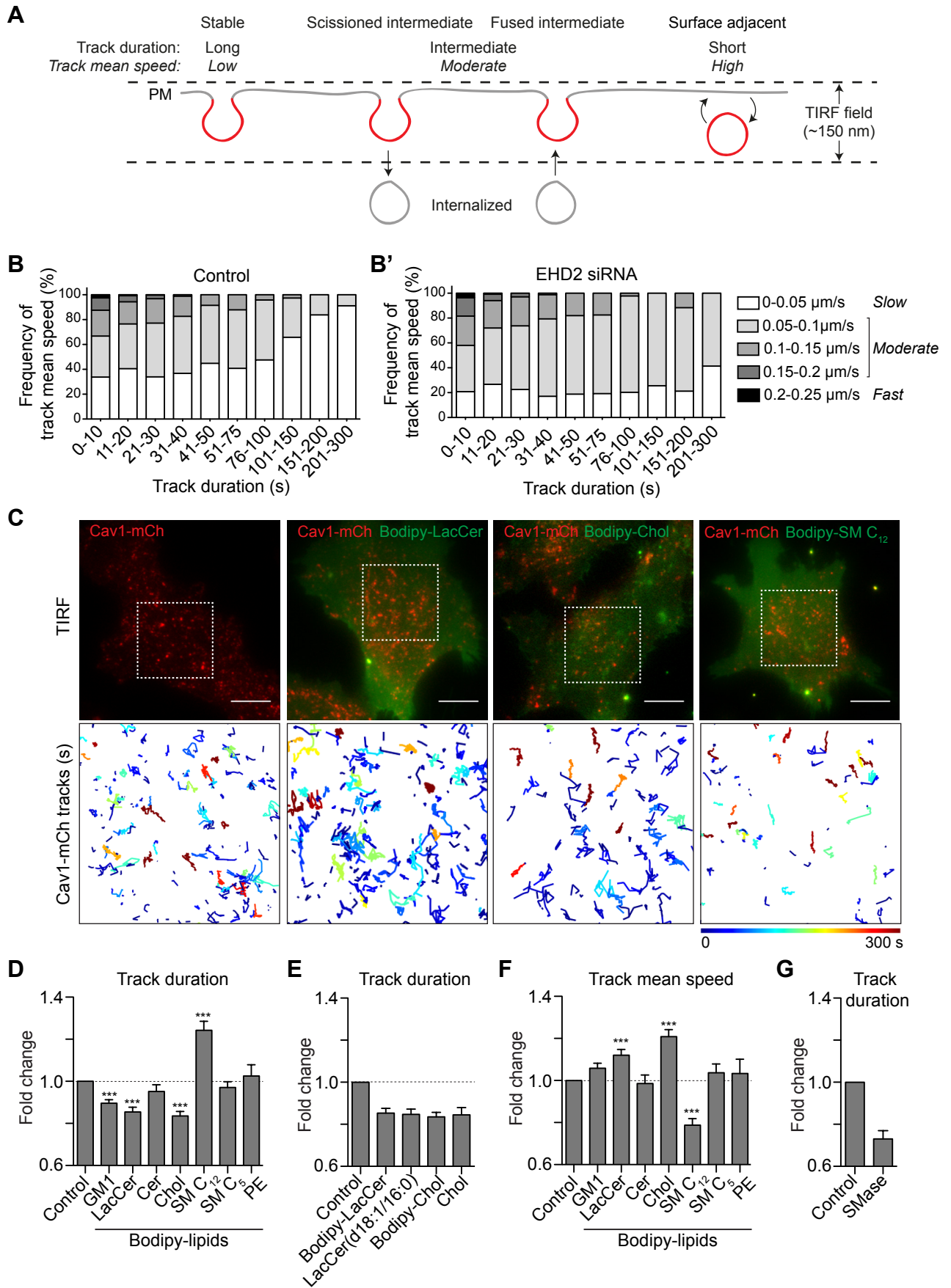
1145 **(A)** Cav1-mCh HeLa cells expressing Rab5-BFP were incubated with Bodipy-labeled LacCer  
1146 or Chol for 15 min. Individual channels are shown for selected areas (dotted box). **(B)**  
1147 Colocalization of lipids with Rab5-positive structures after indicated time-points. **(C)** Cav1  
1148 siRNA-treated Cav1-mCh HeLa cells expressing Rab5-BFP after incubation with Bodipy-  
1149 labeled LacCer or Chol for 15 min. High-magnification images of selected areas (dotted box)  
1150 for each channel are shown. **(D)** Quantification of EE positive for lipids in cells treated with  
1151 siRNA control or against Cav1. Cells were incubated with Bodipy-lipids for 15 min. **(E)**  
1152 Representative immunoblots of Cav1-mCh HeLa cells treated with control siRNA or siRNA  
1153 against Cav1. Clathrin HC served as loading control. **(F)** Cav1-mCh HeLa cells were  
1154 incubated with Bodipy-lipids for 15 min, fixed and LDs were stained using LipidTOX-DR.  
1155 **(G)** Colocalization of lipids to LDs. **(H)** Colocalization of lipids with LDs in cells depleted of  
1156 Cav1 after 15 min. (B, D, E, F)  $n = 10$ , mean + SEM. All scale bars, 5  $\mu\text{m}$ .



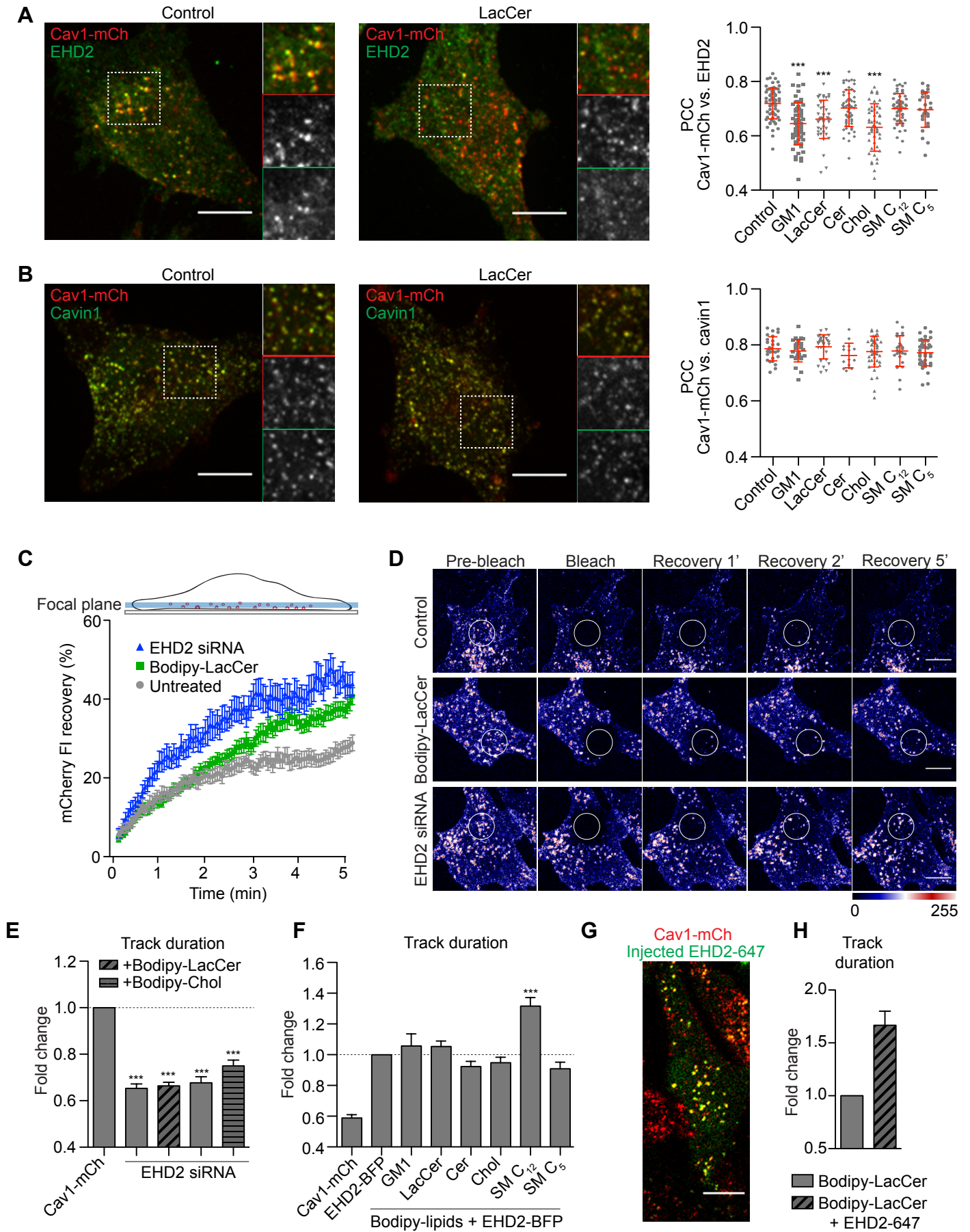
**Figure 1**



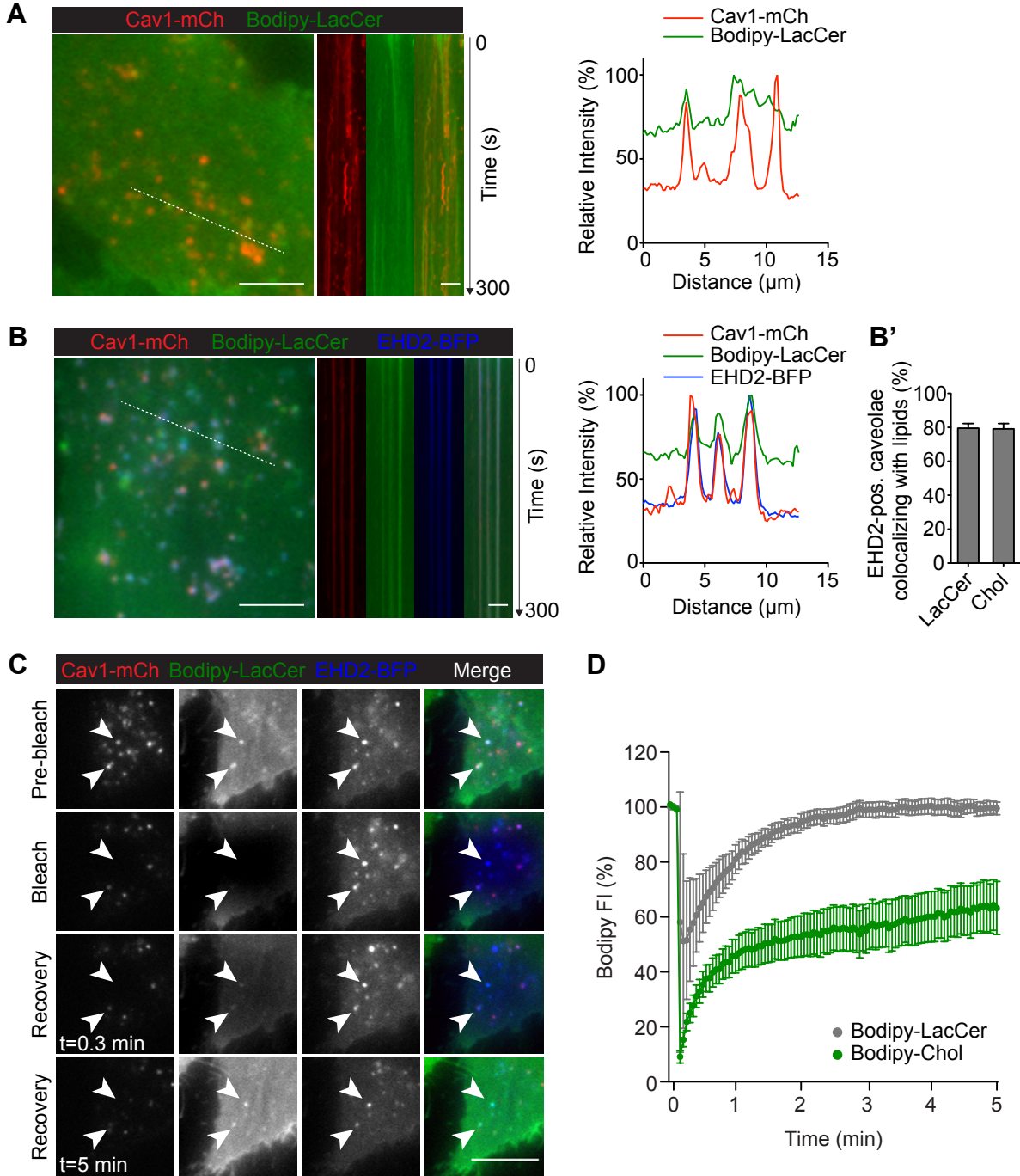
## Figure 2



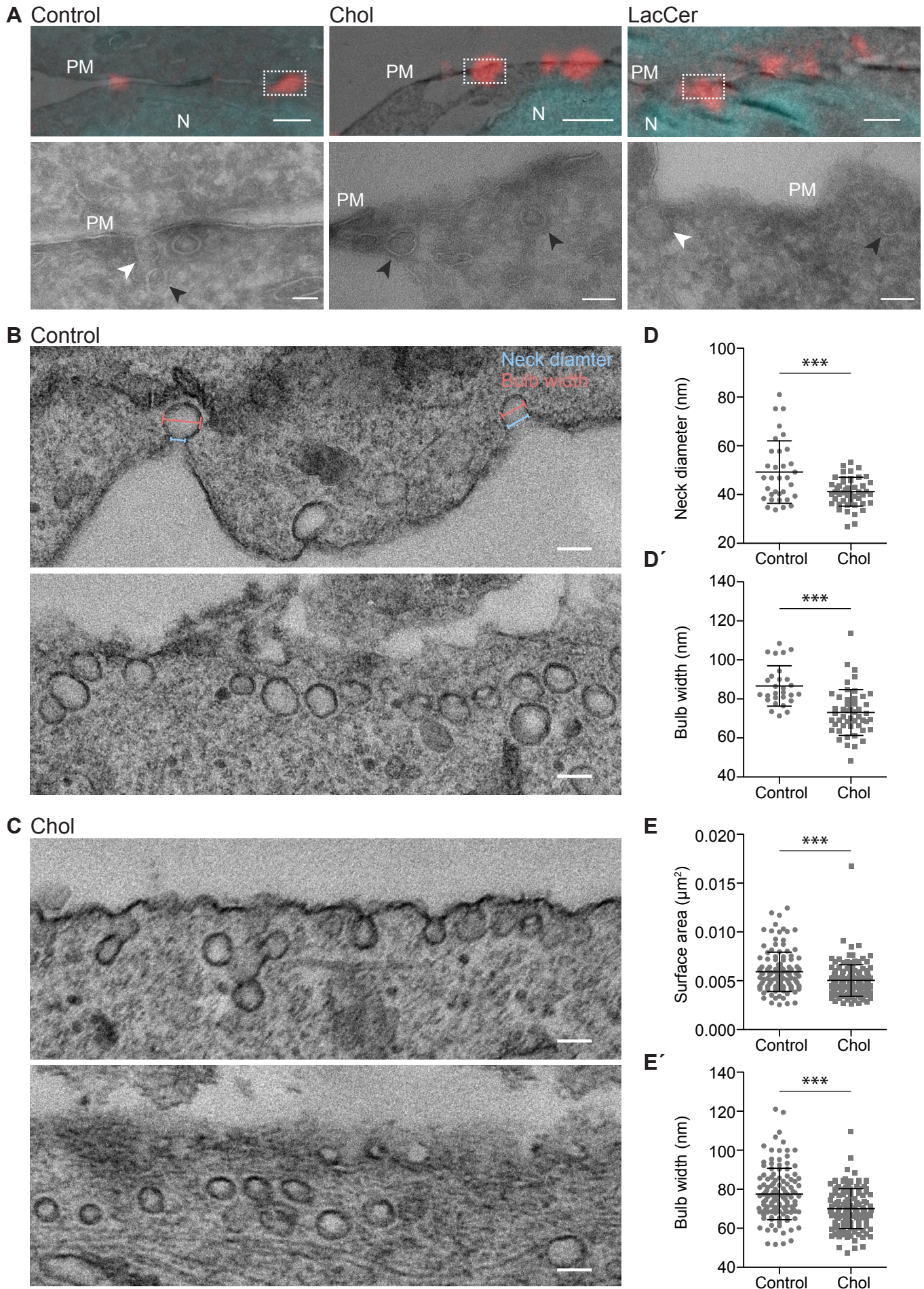
### Figure 3



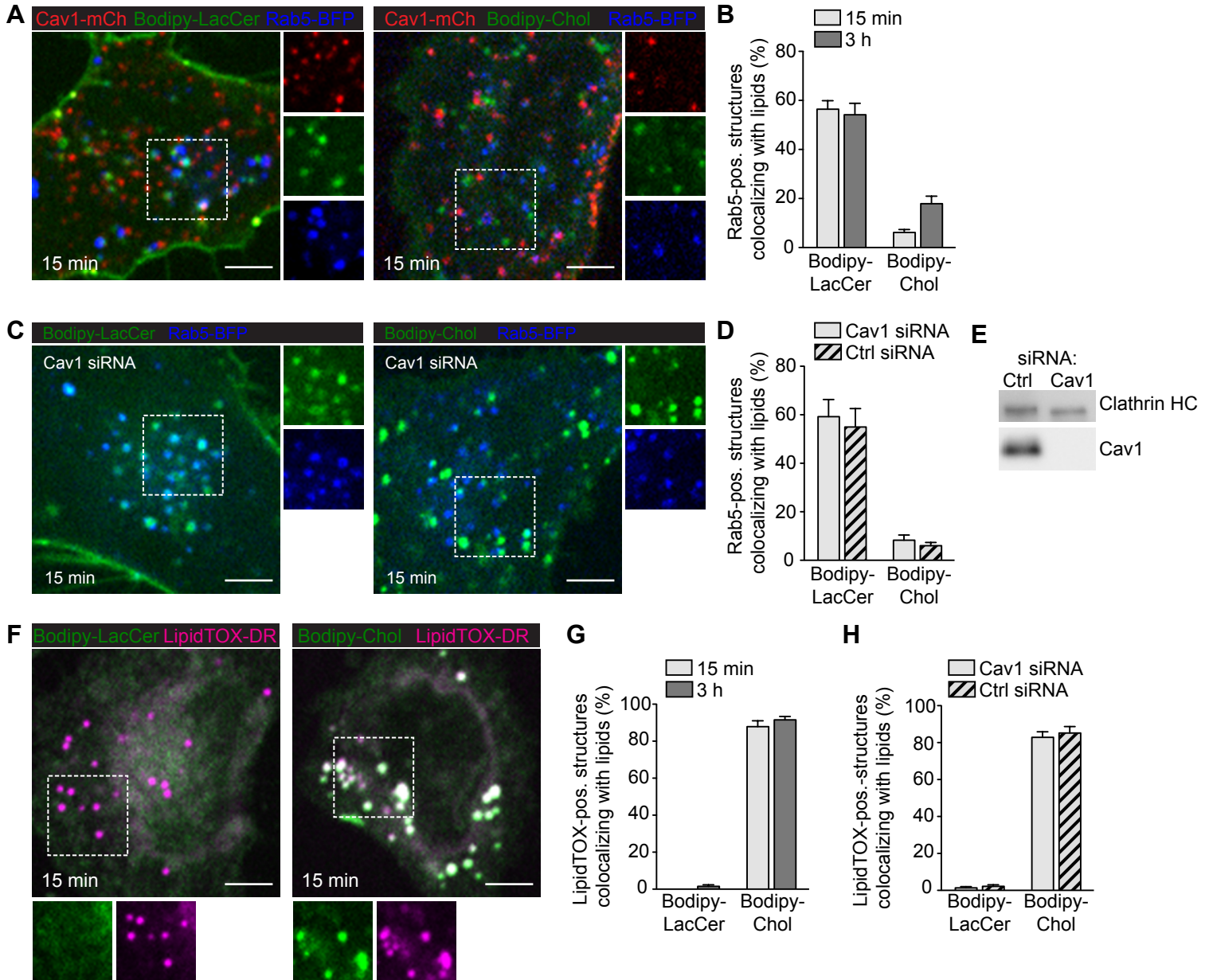
## Figure 4



## Figure 5



## Figure 6



# Supplemental Material

## **Lipid accumulation promotes scission of caveolae**

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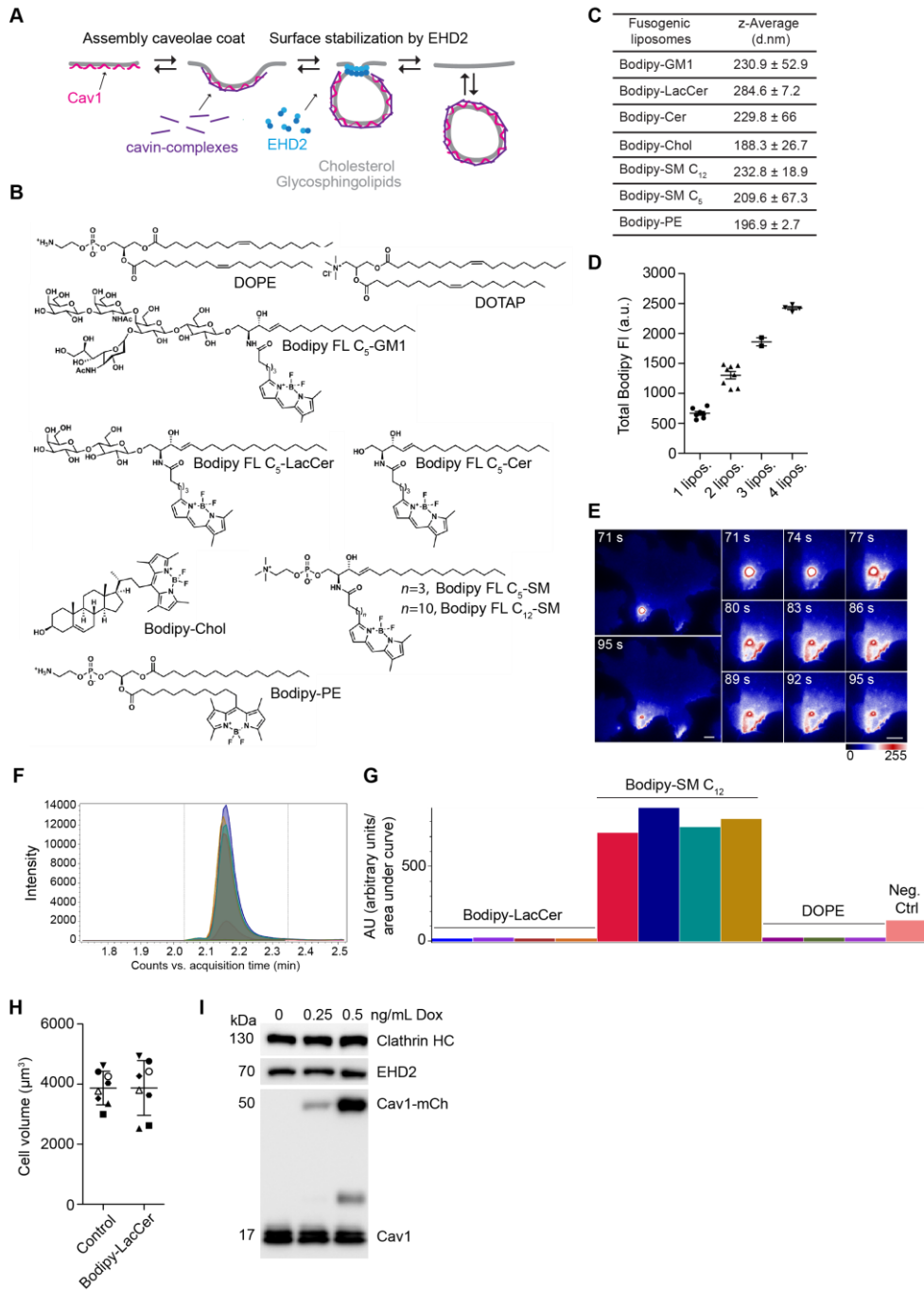
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**Figure S1 (Figure 1- Supplement 1)**

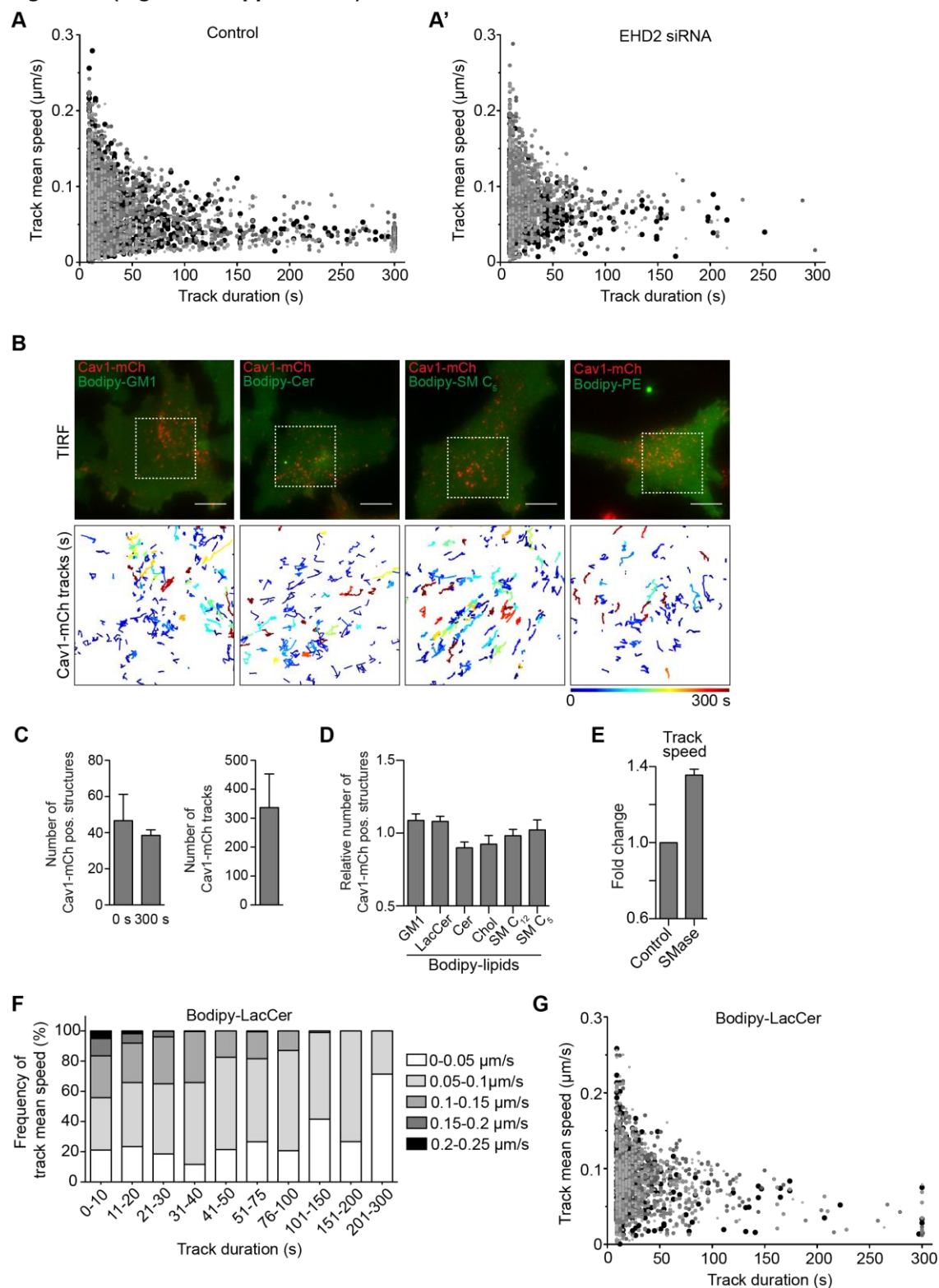


**Fig. S1. Liposome characterization and incorporation efficiencies of Bodipy-lipids measured by mass spectrometry.** (A) Scheme illustrating caveolae dynamics at PM. Caveolae formation and coat assembly are primarily driven by the integral membrane protein Cav1 and cavin proteins. EHD2 controls surface association of caveolae. (B) Chemical structures of lipids used in this study. (C) Hydrodynamic diameter as z-average of DOPE:DOTAP:Bodipy-lipid liposomes.  $n = 3$ , three independent experiments, mean  $\pm$  SD. (D) Total Bodipy FI of liposomes containing Bodipy-LacCer was determined in a single confocal section ( $0.5 \mu\text{m}$ ) using spinning disk microscopy. Bodipy FI corresponds to the number of liposomes measured in each ROI.  $n = 22$ , mean  $\pm$  SEM. (E) Time-lapse imaging of a vesicle fusing with the PM. A single fusion event highlights the rapid distribution of the fluorophore from the liposome-membrane contact site and subsequent fusion. Distribution of Bodipy fluorescence is intensity-coded using LUT. Scale bars,  $10 \mu\text{m}$ . (F, G) Visualization of chromatography of Bodipy-SM C<sub>12</sub>. Samples visualized: Bodipy-LacCer treated samples, Bodipy-SM C<sub>12</sub> treated samples, DOPE control samples and a negative control of Bodipy-SM C<sub>12</sub> (liposomes added to wells without cells). (F) Chromatography of the samples. (G) Integrated area of each individual sample. (H) Analysis of the cell volume before and after addition of Bodipy-LacCer liposomes. Cell surface was segmented with Imaris using mCh fluorescence. Identical symbols in control and Bodipy-LacCer represent the same cell.  $n = 8$ , mean  $\pm$  SD. (I) Representative immunoblots showing protein



expression of EHD2, Cav1-mCh and Cav1 after induction of Cav1-mCh HeLa cells with different concentrations of Dox. Clathrin HC served as loading control. Related to Fig. 1.

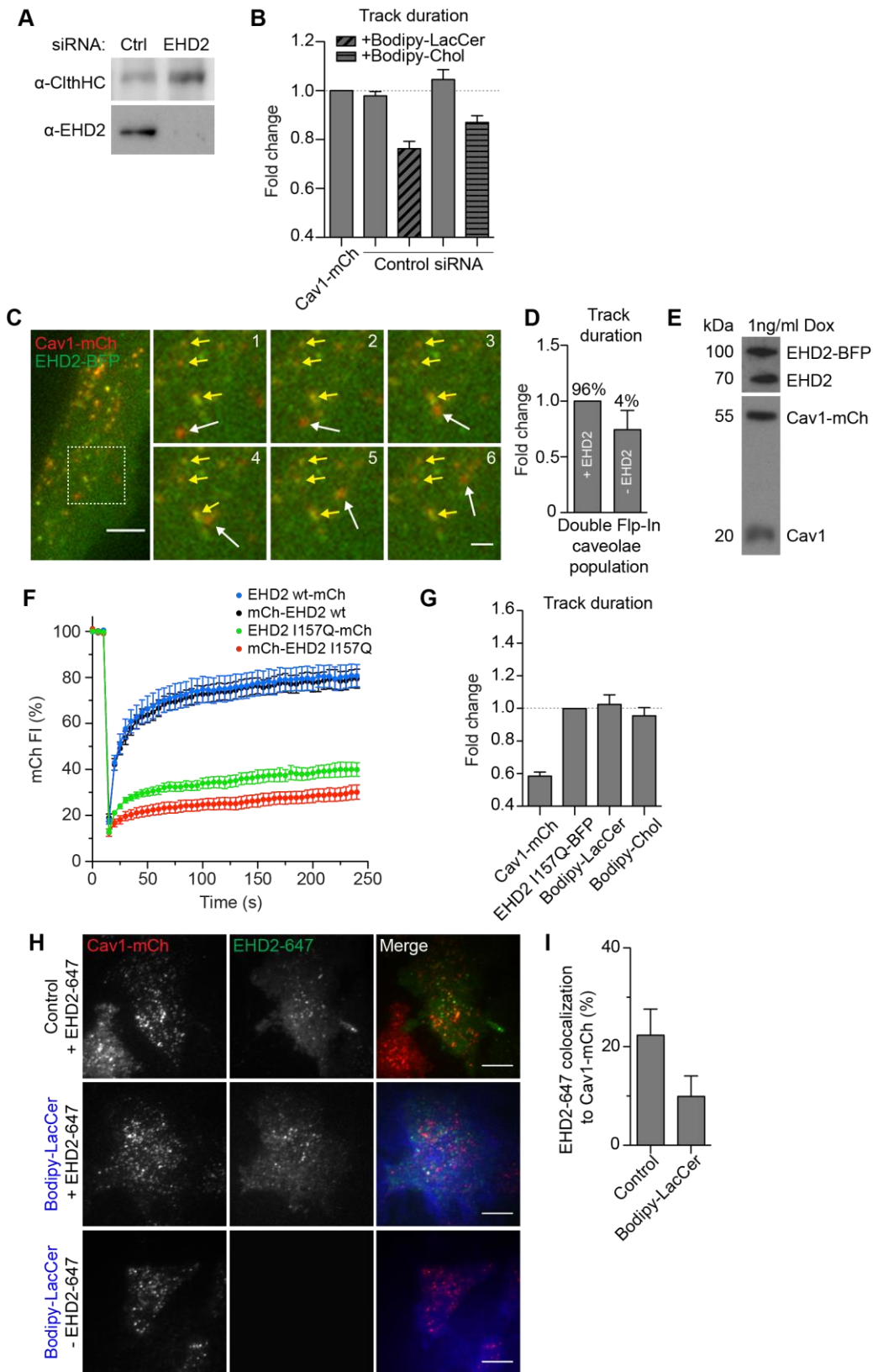
**Figure S2 (Figure 2- Supplement 1)**



**Fig. S2. GSLs and Chol affect lifetime of caveolae while caveolae numbers at the PM are unchanged. (A, A')** Correlation between track duration and track mean speed. TIRF live cell movies of Cav1-mCh structures (A) and cells lacking EHD2 (A') were analyzed (five datasets for each condition). Identical symbols represent tracks from the same cell. (B) Representative images from TIRF live cell movies of Dox-induced Cav1-mCh HeLa cells after incubation with different fusogenic liposomes containing Bodipy-lipids (final total lipid concentration of 7 nmol/mL) for 15 min. Cav1-mCh structures were tracked using Imaris software. Color-coded trajectories illustrate time that structures can be tracked at PM over 5 min (dotted square). Scale bars, 10  $\mu\text{m}$ . (C) Number of Cav1-mCh positive structures at the beginning and at the end of 5 min TIRF movies and the corresponding number of tracks

detected.  $n \geq 8$ , three independent experiments, mean + SEM. **(D)** Relative number of caveolae at the PM of Cav1-mCh HeLa cells before and after addition of fusogenic liposomes. TIRF live cell movies from Fig. 2C and S2B were analyzed. Number of caveolae after lipid treatment was normalized to the number of caveolae in control cells.  $n \geq 8$ , three independent experiments, mean + SEM. **(E)** Quantification of track mean speed of Cav1-mCh structures from TIRF movies following incubation with SMase for 2 h. Fold changes are relative to control (Cav1-mCh).  $n \geq 5$ , mean + SEM. **(F)** Distribution of track mean speed in subpopulations of track duration of Cav1-mCh structures treated with Bodipy-LacCer liposomes. **(G)** Correlation between track duration and track mean speed of Cav1-mCh structures treated with Bodipy-LacCer liposomes. In (F) and (G) five datasets were analyzed. All analysis was performed using Imaris software. Related to Fig. 2.

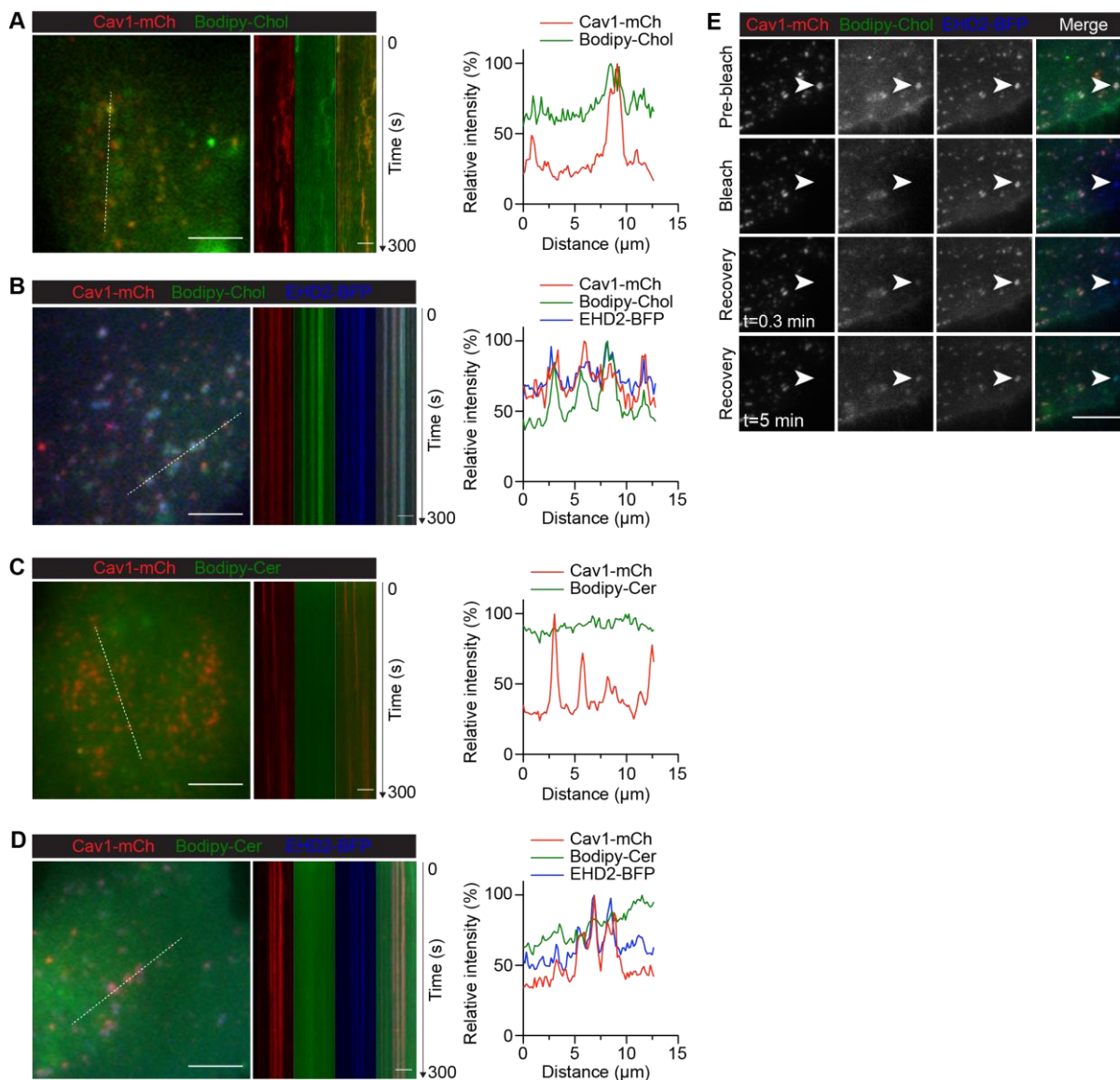
**Figure S3 (Figure 3- Supplement 1)**



**Fig. S3. Stabilization of caveolae to the PM by EHD2 and EHD2-I157Q cannot be reversed by addition of Bodipy-labeled LacCer or Chol.** (A) Representative immunoblots of Cav1-mCh HeLa cells treated with Ctrl siRNA or siRNA against EHD2. Clathrin HC served as loading control. (B) Effect of lipids on track duration of Cav1-mCh structures analyzed following control siRNA-treatment.  $n \geq 8$ , two independent experiments, mean + SEM. (C) Representative time-lapse images of Cav1-mCh positive for EHD2-BFP (yellow arrows) or lacking EHD2-BFP (white arrows) in double Flp-In EHD2-BFP Cav1-mCh HeLa cells. Dotted box shows higher

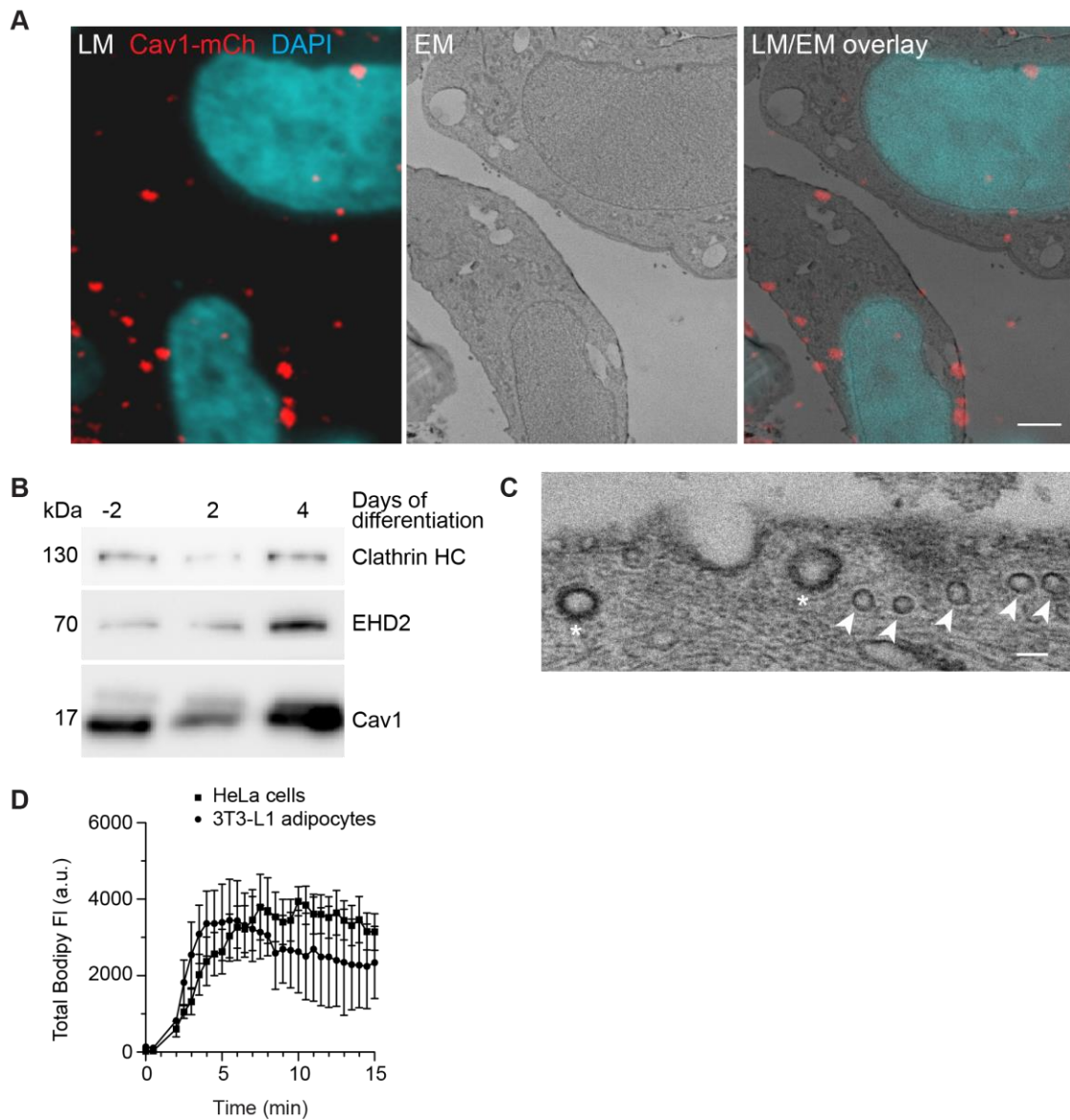
magnification region. Numbering corresponds to number of frames. Scale bar, 10  $\mu\text{m}$ ; inset scale bars, 2  $\mu\text{m}$ . **(D)** Differences in track duration of Cav1-mCh structures positive for EHD2-BFP or lacking EHD2-BFP in double Flp-In EHD2-BFP Cav1-mCh HeLa cells. Percentage of Cav1-mCh structures positive or lacking EHD2-BFP are indicated.  $n = 8$ , mean + SEM. **(E)** Representative immunoblots of double Flp-In EHD2-BFP Cav1-mCh HeLa cells induced with 1 ng/ml Dox. **(F)** FRAP curves of mCh-tagged EHD2 wt or EHD2 I157Q expressing HeLa cells. A ROI was photobleached and recovery of mCherry fluorescence intensity (mCherry FI) was monitored. Intensities were normalized to background and reference.  $n = 8$ , mean  $\pm$  SEM. **(G)** Cav1-mCh HeLa cells transiently expressing EHD2-I157Q-BFP were incubated with Bodipy-LacCer or Bodipy-Chol liposomes and track duration was analyzed.  $n \geq 8$ , two independent experiments, mean + SEM. **(H)** Representative live cell TIRF images of Cav1-mCh HeLa cells untreated or treated with Bodipy-LacCer and with or without microinjection of EHD2-647. **(I)** Quantification of the colocalization of microinjected EHD2-647 to Cav1-mCh in control cells and cells treated with Bodipy-LacCer liposomes prior to injection.  $n \geq 5$ , mean + SEM. Scale bar, 10  $\mu\text{m}$ . In (B, D, G) Imaris software was used to analyze data. Changes in track duration are relative to control (indicated by dotted line). Related to Fig. 3.

**Figure S4 (Figure 4- Supplement 1)**



**Fig. S4. LacCer and Chol but not Cer accumulate in caveolae at the PM and recover within caveolae following photobleaching.** (A, B) Cav1-mCh HeLa cells (A) and Cav1-mCh HeLa cells transiently expressing EHD2-BFP (B) were incubated with Bodipy-Chol liposomes. White lines indicate the location of the kymograph and the corresponding intensity profiles illustrate the colocalization of Bodipy-Chol with Cav1-mCh either alone or in the presence of EHD2-BFP. Intensity profiles are relative to the maximum value for each sample. (C, D) As for (A, B), cells were incubated with Bodipy-Cer. Scale bars, 10  $\mu\text{m}$ ; kymograph scale bars, 5  $\mu\text{m}$ . (E) Bodipy fluorescence recovery experiments to study the accumulation of lipids in caveolae. Cav1-mCh HeLa cells transiently expressing EHD2-BFP were incubated with Bodipy-Chol liposomes for 10 min. Following photobleaching, the fluorescence recovery of the Bodipy signal within caveolae was monitored over time. White arrow highlights surface connected caveolae with accumulated Chol. Scale bars, 5  $\mu\text{m}$ . Related to Fig. 4.

**Figure S5 (Figure 5- Supplement 1)**



**Fig. S5. Expression of EHD2 and Cav1 is upregulated in 3T3-L1 adipocytes.** (A) Cav1-mCh HeLa cells were induced with Dox and transiently expressed EHD2-GFP. Light microscopy image showing localization of caveolae (Cav1-mCh) and nuclei (DAPI) within cells (LM, left panel). Middle panel depicts corresponding EM images. Overlay of LM and EM images shows correlation of fluorescently labeled structures to ultrastructure in same cells (right panel). Scale bar, 2  $\mu$ m. (B) Representative immunoblots showing protein expression of EHD2 and Cav1 during 3T3-L1 differentiation. Clathrin HC served as loading control. (C) Representative electron micrographs of 3T3-L1 adipocytes. Caveolae (indicated by arrow) can be clearly distinguished from clathrin-coated pits (indicated by asterisk). Scale bar, 100 nm. (D) Incorporation rate of Bodipy-Chol into the PM of live 3T3-L1 adipocytes. Cells were treated with fusogenic liposomes (final total lipid concentration 7 nmol/mL). Total fluorescence intensity (FI) of the Bodipy signal was measured within circular ROIs in a confocal section using spinning disk microscopy. Ten ROIs were analyzed using the Zeiss Zen system software.  $n = 2$ , mean  $\pm$  SEM. Related to Fig. 5.

**Video 1. Cell surface dynamics of Cav1-mCh.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells. The image in Fig. 2C (Cav1-mCh) is taken from this movie. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 2.

**Video 2. Cell surface dynamics of Cav1-mCh after treatment with Bodipy-LacCer.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells after 15 min incubation with liposomes containing Bodipy-LacCer. The image in Fig. 2C is taken from this movie. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 2.

**Video 3. Cell surface dynamics of Cav1-mCh after treatment with Bodipy-Chol.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells after 15 min incubation with liposomes containing Bodipy-Chol. The image in Fig. 2C is taken from this movie. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 2.

**Video 4. Cell surface dynamics of Cav1-mCh after treatment with Bodipy-SM C<sub>12</sub>.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells after 15 min incubation with liposomes containing Bodipy-SM C<sub>12</sub>. The image in Fig. 2C is taken from this movie. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 2.

**Video 5. Bodipy-LacCer colocalizes with Cav1-mCh positive structures.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells after incubation with liposomes containing Bodipy-LacCer. The image in Fig. 4A is taken from this movie and corresponds to the ROI highlighted by the white square. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 4.

**Video 6. Bodipy-Chol colocalizes with Cav1-mCh positive structures.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells after incubation with liposomes containing Bodipy-Chol. The image in Fig. S4A is taken from this movie and corresponds to the ROI highlighted by the white square. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 4.

**Video 7. Bodipy-LacCer accumulates in caveolae.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells transiently expressing EHD2-BFP after incubation with liposomes containing Bodipy-LacCer. The image in Fig. 4B is taken from this movie and corresponds to the ROI highlighted by the white square. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 4.

**Video 8. Bodipy-Chol accumulates in caveolae.** A representative TIRF live cell movie of Dox-induced Cav1-mCh HeLa cells transiently expressing EHD2-BFP after incubation with liposomes containing Bodipy-Chol. The image in Fig. S4B is taken from this movie and corresponds to the ROI highlighted by the white square. Movie in real time spans 5 min and was recorded at 3 s intervals. Scale bar, 10  $\mu$ m. Related to Fig. 4.