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Cholesterol esters form supercooled lipid droplets whose nucleation is facilitated by triacylglycerols

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27 **Abstract**

28 Cellular cholesterol can be metabolized to its fatty acid esters, cholesteryl esters (CEs), to be
29 stored in lipid droplets (LDs). With triacylglycerols (TGs), CEs represent the main neutral
30 lipids in LDs. However, while TG melts at $\sim 4^{\circ}\text{C}$, CE melts at $\sim 44^{\circ}\text{C}$, raising the question of how
31 CE LDs form in cells. Here, we show that CE forms supercooled droplets when the CE
32 concentration in LDs is above 20% to TG and, in particular, liquid-crystalline phases when the
33 fraction of CEs is above 90% at 37°C . In model bilayers, CEs condense and nucleate droplets
34 when the CE/phospholipid ratio reaches over 10-15%. This concentration is reduced by TG
35 pre-clusters in the membrane that thereby facilitate CE nucleation. Accordingly, blocking TG
36 synthesis in cells is sufficient to strongly dampen CE LD nucleation. Finally, CE LDs emerged
37 at seipins, which cluster and nucleate TG LDs in the ER. However, when TG synthesis is
38 inhibited, similar numbers of LDs are generated in the presence and absence of seipin,
39 suggesting that seipin controls CE LD formation via its TG clustering capacity. Our data point
40 to a unique model whereby TG pre-clusters, favorable at seipins, catalyze the nucleation of
41 CE LDs.

42

43

44 **Keywords:** cholesterol ester, lipid droplet biogenesis, supercooling, smectic liquid crystals,
45 nucleation, cholesterol, triacylglycerol

46

47

48 **Introduction**

49 From plant to human, dozens of neutral lipids can be made by cells and deposited into lipid
50 droplets (LDs)(1). LDs control cellular energy and lipid homeostasis but also possess several
51 other functions(2) attuned to the LD formation triggering cue(2–4). LD formation is initiated
52 when sufficient amounts of neutral lipids accumulate in the endoplasmic reticulum (ER)(5,
53 6). As neutral lipids are often apolar and hydrophobic, they are released in the hydrophobic
54 core of the ER bilayer(1). There, neutral lipids condense and nucleate a nascent LD which
55 grows by acquiring more neutral lipids and buds off as a spherical LD out of the ER bilayer(7).
56 Despite many advances in understanding LD biogenesis(8–13, 13, 14), the molecular
57 mechanisms underlying the different steps of LD formation are not fully resolved.

58

59 Triacylglycerols (TGs) are the major neutral lipids in mammalian cells and, *in silico*, they can
60 condense and nucleate a droplet in a bilayer if their ratio to phospholipids exceeds 3-4%(15–
61 17). In cells, this concentration is altered by several lipid and protein factors, including
62 seipin(5, 8). Seipin is an integral ER protein, forming an oligomeric donut shape(10, 18–20)
63 and displaying favorable interactions with TG. Seipin decreases the TG nucleation
64 concentration to ~1.25% (10, 13, 14) to induce nascent LD formation and growth(21–23). In
65 seipin deletion, more TG LDs are nucleated (11, 24, 25) but they fail to grow normally (12).
66 Thereupon, seipin ensures that only a few TG LDs are nucleated and mature properly(1, 12).

67

68 Cells can make other neutral lipids such as squalene, an intermediate in the cholesterol
69 biosynthesis pathway. Squalene alone fails to form LDs in yeast and accumulates in the ER
70 and *in vitro* membranes(16, 26, 27). Therefore, squalene seems to have a higher nucleation
71 concentration than TG. Retinyl esters represent another neutral lipid class found in liver
72 stellate cells. In yeast mutant cells lacking neutral lipids, the biosynthesis of retinyl palmitate,
73 induced by the overexpression of the lecithin retinol acyltransferase, leads to the formation
74 of retinyl palmitate LDs(28), which can form away from seipin's location(28). Seipin may
75 therefore preferentially act on some neutral lipids(1, 16). How exactly neutral lipids impact
76 LD formation and whether/how seipin is involved in the nucleation of LDs composed of
77 neutral lipids other than TG remain unknown.

78

79 Together with TGs, cholesterol esters (CEs) represent the most abundant neutral lipid in
80 mammalian cells. TG results from diacylglycerol esterification and CE from cholesterol
81 esterification to a fatty acid. Depending on the cell type, TG/CE ratios vary significantly. For
82 instance, white adipocytes, specialists in long-term energy storage, can make an ultra-large
83 TG-rich LD, tens of μm in diameter. Macrophages can make CE-rich LDs and convert to foam
84 cells during atherogenesis. In some cells, mixed TG/CE LDs are generated while in others,
85 such as adrenocortical cells, specialized in steroid hormone synthesis, spatially distinct TG
86 and CE LDs can form(29).

87

88 TGs and CEs are chemically highly divergent and therefore might require different
89 membrane physical chemistry and protein settings to be packaged into LDs. For instance,
90 triolein (TG) melts at $\sim 4^\circ\text{C}$ while cholesterol oleate (CE) only melts at $\sim 44^\circ\text{C}$, and TG escapes
91 curved membrane regions in contrast to CE (11, 27). Whether CE molecules can condense
92 into a forming LD in the ER bilayer as TGs do and whether seipin mediates the formation of
93 CE LDs similarly as it does for TG LDs, is unclear. We addressed these questions in this
94 manuscript.

95

96 **Results**

97 **CEs form supercooled liquid droplets**

98 Since the CE melting point is $\sim 44^\circ\text{C}$, we asked whether CE can be emulsified at 37°C . We
99 heated CE in a test tube to 37°C and it remained solid as one would expect. At 50°C , it
100 formed a liquid phase but solidified when the temperature was brought back to 37°C (Figure
101 1A). At 37°C , adding a buffer phase and mixing was insufficient to emulsify the CE powder
102 (Supplementary Figure 1A). Thus, bulk CE cannot remain at equilibrium in a liquid state at
103 37°C .

104
105 Next, we liquefied the CE sample and added the buffer at 50°C . Vortexing the mixture
106 allowed us to generate micrometer-sized droplets. Remarkably, when the emulsion was then
107 cooled to 37°C (or to 25°C), the droplets stayed liquid (Figure 1A). The droplets were liquid
108 since they splashed at the water-air interface (Figure 1B). Also, at the water-air surface, they
109 displayed onion rings, features reminiscent of unstable liquids; for comparison, TG did not
110 show such features (Supplementary Movies 1,2). These observations indicated that the CE
111 droplets were trapped in local minimum energy, below their melting point. Such a physical
112 phenomenon is known as supercooling: trapping of liquids in metastable states between
113 their liquid and stable solid states (30). In solution, our generated CE droplets were short-
114 lived, less than 72 h under rotation, as they fused, grew, and crystallized. Instead, when we
115 generated the droplets in the presence of phospholipids to cover their interface with water,
116 fusion and crystallization were prevented and the lifetime of the droplets was prolonged for
117 more than ten days (Supplementary Figure 1B).

118
119 The crystallization of supercooled liquids can be triggered by nucleation seeds (30).
120 Accordingly, when CE droplets met solid seeds at the air-water interface, they crystallized
121 (Figure 1C, Supplementary Movie 3). This observation confirms that the CE droplets were
122 indeed supercooled and explains why we were not able to make ultra large millimetric CE
123 droplets (in contrast to TG). Indeed, droplets of larger size crystallized more readily and had
124 a higher probability to exit supercooling(30). Finally, the stability of supercooled droplets
125 depends on the nature of interfacial interactions(30). By replacing the water phase with
126 silicone oil, we lost the supercooled state, as droplets developed spikes at their interface and
127 crystallized (Supplementary Figure 1C). This observation suggests that interaction of CE with

128 water molecules at the droplet interface contributed to the supercooled state of the
129 droplets.

130

131 Since CE molecules may form anisotropic or liquid crystalline phases(31–34), we prepared CE
132 droplets and imaged them under polarized light, which can reveal such organization. At 25°C
133 or 37°C, we observed a mosaic of internal organizations of CEs in the droplets. The most
134 frequent phenotype was droplets with Maltese crosses (Figure 1D,E, Supplementary Figure
135 1D). Such a signal was indicative of a smectic liquid crystalline phase with an azimuthal
136 organization of CE molecules(35), similar to the liquid crystalline lattices or “onion rings”
137 seen by electron microscopy(33, 34, 36). We also observed isotropic signals indicating no
138 clear internal organization, but this phenotype was less frequent than the crystalline one
139 (Figure 1E). Other droplets had intermediate signals between liquid crystalline and isotropic
140 signatures, or uninterpretable organization (Figure 1D,E, Supplementary Figure 1D). Since
141 the liquids are metastable, these variable organizations likely depend on the preparation
142 method. In any case, pure CE droplets may have different internal organizations but the
143 liquid crystalline state dominates.

144

145 Of note, the physical state of the CE droplets is defined by their free energy state which
146 depends on several parameters such as temperature, pressure, and chemical potential
147 (Supplementary Figure 1F). Here, we tuned the temperature to reach the energy of the
148 supercooled state. Evidently, mammalian cells would not tune this parameter but rather
149 modulate the chemical environment. In any case, regardless of the energetic path taken, our
150 data show that pure CE droplets can be generated under a supercooled state, particularly
151 facilitated by interfacial interactions with water (Supplementary Figure 1C). They are mainly
152 trapped in the supercooled smectic liquid crystalline regime. As a consequence, the larger
153 the CE droplet, the higher its propensity to crystallize.

154

155 **The appearance of the liquid crystalline phase in CE LDs depends on TG/CE ratio**

156 We studied how the physical state of the CE droplets is impacted by TG. In bulk, CE was
157 insoluble in TG (or diacylglycerol) above ~20% molar ratio at 37°C (Supplementary Figure
158 1E,G). When droplets were directly made at 37°C with CE/TG mixtures above 20%, we
159 observed a de-mixed CE crystal and a TG liquid phase (Supplementary Figure 1E,G). Only

160 when the sample was heated to liquefy the blend at 50°C droplets could be made, as above,
161 before the temperature was cooled to 37°C. Consequently, CE/TG mixtures can form stable
162 liquid droplets when CE is below ~20% and become supercooled above this ratio.

163

164 We then imaged the droplets under polarized light upon heating the sample from 30°C to
165 37°C. As expected, below 20% CE, the droplets were isotropic. Above 20%, they were also
166 mostly isotropic, despite being supercooled, and displayed a smectic liquid crystalline phase
167 above ~80% at 30°C and ~93% at 37°C (Figure 1F, Supplementary Figure 1H). These results
168 indicate that the TG/CE ratio tuned the physical state of the droplets and that the liquid
169 crystalline regime is reached whenever CE goes above ~80-90%.

170

171 We next probed whether the CE/TG ratio can modulate the state of cellular LDs, by taking
172 advantage of the liquid crystalline signature upon polarized light illumination. We employed
173 A431 cells and loaded them with 200 μM cholesterol complexed with methyl-β-cyclodextrin
174 (MβCD) for 24 h to make CE-rich LDs, and then imaged the cells at 37°C (Figure 1G,
175 Supplementary Figure 1I). We found 95% of the LDs to display a Maltese cross signal, the
176 smectic liquid crystalline signature, suggesting that they were more than 90% enriched in CE.
177 To vary the TG level, we supplemented the cells with both oleic acid and cholesterol at 20/80
178 (Figure 1G, Supplementary Figure 1J). We found a significant decrease in the number of LDs
179 in the liquid crystalline state (Figure 1H, Supplementary Figure 1J,L,M). This result argues
180 first that A431 cells generated mixed LDs containing both CEs and TGs and second, that in
181 the presence of oleic acid, LDs had increased TG levels, which disrupted the liquid crystalline
182 organization, in line with our *in vitro* experiment (when TG/CE levels are above ~1/9 at 37°C;
183 Figure 1F). Accordingly, the liquid crystalline phase phenotype of LDs was restored in cells
184 loaded with both oleic acid and cholesterol in the presence of pharmacological inhibitors of
185 diacylglycerol O-acyltransferase 1 and 2 enzymes (DGAT1,2) that synthesize TG
186 (Supplementary Figure 1K,L).

187

188 Together, these data indicate that the TG/CE ratio determines the molecular organization of
189 LDs. Based on our *in vitro* studies, LDs would be under stable conditions when the CE
190 concentration in LDs is below 20% relative to TG at 37°C; at above 20% CE, LDs would be in a
191 supercooled state and, particularly, in a smectic liquid crystalline phase at above 90% CE.

192

193 **Inhibition of TG synthesis compromises and stimulation of TG synthesis enhances CE LD**
194 **formation in cells**

195 As TG determined the physical states of CE-containing LDs, we asked whether it has a role in
196 the biogenesis of such LDs. We, therefore, tested if inhibition of TG synthesis affects CE LD
197 formation in A431 cells loaded with increasing amounts of cholesterol from M β CD for 1 h
198 when LDs appeared and CE levels increased (Supplementary Figure 2A,B). This revealed that
199 inhibition of DGAT1+2 activity during cholesterol loading strongly compromised CE LD
200 formation. At 50 μ M cholesterol, while few LDs were generated in WT cells, almost none was
201 made in the DGAT-inhibited condition (Figure 2A,B). This observation argues that CE LD
202 nucleation was enhanced by ongoing TG synthesis, despite the fact that no OA was added to
203 the medium. At higher concentrations, i.e., 100 and 200 μ M cholesterol, LDs were made in
204 both WT and DGAT-inhibited conditions, but the number and integrated size of LDs were
205 significantly lower in the latter case (Figure 2B, Supplementary Figure 2A-C). These data
206 support the hypothesis that CE LD nucleation is facilitated by ongoing TG synthesis, even
207 though no OA was supplied to the cells.

208

209 Based on the above observations, we investigated if spiking cells with small amounts of OA
210 during cholesterol loading might ease CE LD formation by TG synthesis. While 100 μ M
211 cholesterol alone for 30 min did not efficiently induce LD formation (Figure 2C,
212 Supplementary Figure 2D, E), adding 10 μ M OA together with 100 μ M cholesterol increased
213 CE LD amounts more than what was achievable by 100 μ M cholesterol or 10 μ M OA alone
214 (Figure 2C, Supplementary Figure 2D). The effect was even more pronounced with 20 μ M
215 OA, which when spiked together with 100 μ M cholesterol, increased the number of CE LDs
216 more than 2-fold (Figure 2C, Supplementary Figure 2D-F). Importantly, the effect of OA
217 spiking was sensitive to DGAT inhibitors indicating that the increased number of LDs was
218 achieved via enhanced TG synthesis. These experiments strongly support the idea that
219 ongoing TG synthesis mediates the efficient nucleation of CE LDs.

220

221 To further validate these findings, we used a long cholesterol loading time to enable the
222 formation of large LDs that could be visualized under polarized light. A431 cells were fed
223 with 200 μ M cholesterol for 24 h, with or without DGAT inhibitors (Figure 2D). The number

224 of LDs under the crystalline phase was unchanged (Figure 2D, 2F), i.e. LDs were more than
225 90% CE rich at 37°C (Figure 1F, Supplementary Figure 2G). However, the number of such LDs
226 was decreased by almost half in the DGAT-inhibited condition (Figure 2G), although no OA
227 was added. This result is consistent with the decrease in the number of nucleated LDs in the
228 presence of DGAT inhibitors (Figure 2A-C). Next, we modified the experiment by loading with
229 190 μ M cholesterol spiked with 10 μ M OA, representing 5% of the load (Figure 2E). The LDs
230 generated still exhibited the crystalline organization (Figure 2F, Supplementary Figure 2I),
231 indicating that the level of OA supplied and the TGs subsequently made did not alter the
232 physical state of the LDs. Consistently with TG spiking in the nucleation experiment (Figure
233 2C, Supplementary Figure 2D), 5% OA supply was sufficient to significantly increase the
234 number of CE LDs (Figure 2G, Supplementary Figure 2H). Moreover, the presence of DGATi
235 did not change the physical state of the LDs but decreased the number of LDs to a similar
236 level as without OA (Figure 2G).

237

238 Our data indicate that ongoing TG synthesis determines the efficiency of CE LD formation. To
239 rule out that these findings were specific to the A431 cell line, we also analyzed Cos7 and
240 HeLa cells, loaded with cholesterol or cholesterol and OA. There, we likewise found that the
241 inhibition of DGATs significantly reduced the number of LDs (Supplementary Figure 2J-O).

242

243 **CE LDs nucleate at 10-15% to membrane phospholipids**

244 Based on the above data, we hypothesized that CE molecules might not efficiently condense
245 into droplets in membranes and that TGs could assist in this step. We next investigated the
246 potential principles underlying this phenomenon.

247

248 To investigate the behavior of CEs in a simple bilayer, we mixed dioleoylphosphatidylcholine
249 (DOPC) and dioleoylphosphatidylethanolamine (DOPE) phospholipids (70/30), reported by
250 rhodamine-PE at 1%, and cholesteryl oleate (CE), reported by cholesteryl linoleate-NBD at
251 1%. The mixture was then dried and hydrated to make giant unilamellar vesicles (GUVs) with
252 different CE/phospholipid ratios by electroformation. Up to 10% CE, we observed a uniform
253 CE signal in membranes (Figure 3A, Supplementary Figure 3A). Only at a concentration of 15-
254 20%, CE droplets were seen in the GUV bilayer (Figure 3A, Supplementary Figure 3A). To
255 validate this observation, we burst the GUVs on a glass coverslip to better visualize the

256 membrane and the droplet, in 2D. Only starting at 15%, we detected the presence of CE
257 droplets in the membrane (Figure 3B). The droplet signal recovered after photobleaching,
258 indicating that the droplet was in equilibrium with CE molecules in the bilayer (Figure 3C).
259 Accordingly, above 15%, the concentration of free CE in the bilayer was almost constant, the
260 excess being likely adsorbed by the nucleated droplet (Figure 3D); in parallel, the frequency
261 of GUVs containing droplets sharply increased at 15% (Figure 3E). These data indicate that
262 CE droplets nucleate in DOPC/DOPE (70/30) bilayers at 15-20% concentration of
263 CE/phospholipids.

264

265 To further investigate the molecular details of CE nucleation, we examined the process using
266 atomistic molecular dynamics simulations. We performed simulations with varying
267 concentrations of CE (5 mol%, 7 mol%, and 10 mol%) in phospholipid bilayers having a lipid
268 composition similar to that of the ER (see Methods and ref. (14)). In line with the GUV
269 experiments, we failed to observe a stable association between the CE molecules at 5-7
270 mol% concentration (Figure 3F-G). However, when the CE concentration in the bilayer was
271 increased to 10 mol%, we observed CE molecules to form stable aggregates (Figure 3F-G).
272 Similar CE clustering was observed at higher CE concentrations in DOPC/DOPE (70/30)
273 bilayers (Supplementary Figure 3D), which matches the lipid composition used in the
274 experiments. The arrangement of CE molecules in these aggregates was such that primarily
275 the sterol rings were stacked against each other (Supplementary Figure 3B-C), which is
276 reminiscent of the liquid crystal organization.

277

278 **TGs facilitate CE clustering and LD formation in the bilayer**

279 The nucleation concentration for CE, 10-15%, is much higher than the one observed for TG,
280 3-4%, for a similar bilayer composition (15–17). Thus, CE condenses with more difficulty into
281 droplets than TG. To investigate if CEs can be favorably incorporated into a nascent TG
282 droplet in a bilayer, we generated GUVs containing 10 mol% of CE molecules, i.e. when no
283 CE droplet formed (Figure 3D-E). Then, we incorporated artificial droplets to make droplet-
284 embedded vesicles (37, 38) with different neutral lipid compositions and determined the
285 partitioning of CEs between the bilayer and the droplet (Figure 4A). We found that CEs
286 partitioned more favorably into TG droplets than diacylglycerol or squalene droplets (Figure
287 4B, Supplementary Figure 4A). This analysis suggests that CE LD assembly could be

288 particularly helped by TG pre-clusters and not DAG clusters. In agreement with this
289 conclusion, the inhibition of DGATs which accumulates DAG, was inhibitory to LD nucleation
290 (Figure 2).

291

292 To directly test our hypothesis, we used all-atom molecular dynamics simulations to capture
293 nucleation in the case of TG/CE mixtures. We explored the mixing process of CEs with TGs in
294 a realistic ER bilayer containing both TGs and CEs at varying concentrations. At low
295 equimolar concentrations of both neutral lipid species (2.5 mol% CE + 2.5 mol% TG), we did
296 not observe the formation of a stable cluster by either of them (Figure 4C). However, when
297 the concentration of TG was raised to 3 mol% and CE to 4 mol%, TG molecules began to form
298 a stable cluster with CEs (Figure 4C), with CE and TG molecules interspersed in the cluster. At
299 a higher concentration (5.0 mol% CE + 5.0 mol% TG), a larger fraction of CE molecules
300 clustered with TGs (Figure 4C). Analysis of the relative populations of different species
301 showed that the fraction of CEs clustering with TGs increased during the mixing process
302 (Figure 4D), while the fraction of free CE monomers decreased (Supplementary Figure 4B,C).
303 For comparison, very little change was observed in the fraction of CEs embedded in TG-free
304 clusters of CEs (Supplementary Figure 4D), suggesting that TGs facilitate the incorporation of
305 CEs into neutral lipid droplets. Interestingly, while at 7% CE nucleation did not happen
306 (Figure 4E), a mixture of 3%TG and 4%CE (a total of 7% of neutral lipids), allowed the
307 nucleation of CE-containing droplets (Figure 4E). Altogether, these data argue that it is more
308 difficult to incorporate a CE molecule in a pure CE pre-cluster than in a TG pre-cluster, which
309 can explain the difficulty of nucleation in the case of CE. Hence, TG pre-clusters catalyze the
310 nucleation of CE droplets better than CE pre-clusters.

311

312 **TG synthesis is required for the efficient nucleation of CE LDs by seipin**

313 Since seipin clusters TGs, thereby presumably controlling the nucleation of TG-rich LDs, and
314 TG clusters stimulate CE LD nucleation, we speculated that CE LDs should preferentially form
315 at seipins as well. To address this, we employed A431 cells with endogenously tagged seipin-
316 GFP and loaded them with cholesterol from M β CD. We found that seipin colocalized with
317 the formed CE LDs (Figure 5A). To assess if seipin affects the sites of CE LD formation, we
318 employed A431 cells where endogenous seipin was trapped in the nuclear envelope (12). In
319 these cells, CE LDs formed more readily at this ER subdomain (Figure 5B, Supplementary

320 Figure 5A). Together, these results indicate that seipin associates with CE LDs and can
321 control the sites of CE LD formation in A431 cells.

322

323 *In silico* experiments indicate that, mechanistically, seipin controls TG LD nucleation by
324 decreasing the nucleation concentration of TGs from 3-4% to 1.25% of bilayer lipids, via the
325 interaction of TGs with seipin transmembrane and luminal helices (9, 10, 13–16). We
326 therefore investigated if seipin also interacts with CEs using atomistic simulations. In a model
327 ER bilayer with the seipin oligomer (luminal domain + transmembrane region) and 5 mol%
328 CE randomly distributed around it, we observed diffusion of CEs into the lumen of the seipin
329 oligomer and subsequent interaction of the carbonyl group with residue S166 or S165 in the
330 membrane-embedded $\alpha 2$ - $\alpha 3$ helices (Figure 5C,D, Supplementary Figure 5B), previously
331 reported as TG interaction sites (13, 14) and recently found also to interact with CE(39).
332 Mutations of these residues to A abrogated the interaction (Figure 5E), as shown for TGs (13,
333 14). We further observed incoming CE molecules to interact with a seipin-bound CE and to
334 form dimers (Supplementary Figure 5B), reminiscent of the CE arrangement in the liquid
335 crystalline phase.

336

337 We then asked if the interaction strengths of seipin with TG and CE differ, using a long
338 timescale (5 μ s) simulation of only the seipin oligomer luminal domain embedded in a model
339 ER bilayer with 2.5 mol% TGs and 2.5 mol% CEs. This showed that TG and CE interacted with
340 S166 residues on different protomers (Supplementary Figure 5B). However, we did not
341 observe instances of one neutral lipid species displacing the other from its S166-bound state.
342 We also performed umbrella sampling atomistic molecular dynamics simulations to calculate
343 the free energies of TG-S166 and CE-S166 interactions. This revealed that, within the error
344 bars, TG and CE manifest similar free energy profiles (Supplementary Figure 5C): the bound
345 state (about 0.3 nm) is separated from the bulk state by a barrier of about 8 kJ/mol.
346 Therefore, the binding affinity of TG and CE with these key residues is essentially similar.

347

348 The above results indicate that seipin can similarly interact with TG and CE and, therefore,
349 one might expect TG and CE formation in cells to occur rather similarly at seipin defined
350 sites. However, although TG and CE could interact similarly with seipin, a condensation step
351 must occur for the molecules to subsequently form a nascent droplet. Such condensation

352 happens efficiently for TG, at roughly 2-3 mol%(14–17), while for CE it happened only at 10-
353 15 mol% (Figure 3) in model membranes. Therefore, in the presence of seipin, the clustering
354 of TGs at seipins could facilitate the subsequent condensation and nucleation of CE LDs.

355

356 To disentangle the impact of TGs and seipin in the condensation of CEs into LDs, we
357 investigated cells under conditions where either TG synthesis was blocked and/or seipin
358 removed. We have earlier reported that when A431 cells were loaded with OA, first TG LDs
359 appeared within a few minutes (12) and in 20 min, tens of droplets were detectable OA
360 concentration-dependently, with up to ~50 LDs at 200 μ M OA (14). However, when we
361 loaded A431 cells with cholesterol from M β CD with similar lipid concentrations, CE LDs
362 appeared considerably more slowly, with ~50 droplets detectable only in 1 h using 200 μ M
363 cholesterol (Figure 5F-G). Interestingly, when seipin knockout cells were similarly loaded
364 with cholesterol, the initiation of new LDs required a higher cholesterol concentration (100
365 μ M vs. 50 μ M in WT cells) but at 200 μ M cholesterol, the LD numbers were essentially
366 similar to WT cells. Moreover, the numerous tiny LDs characteristic of seipin knockout cells
367 upon OA loading (~250 LDs with 200 μ M OA in 1 h, (14)) were not observed upon cholesterol
368 loading (Figure 5F-G). These data argue that despite the ability of seipin to interact with CEs
369 and position CE LDs in cells, CEs behave fundamentally differently from TGs in the ER, and
370 that this contributes to CE LD formation in cells.

371

372 Finally, we analyzed the effect of inhibiting DGATs during CE LD formation in seipin deficient
373 cells. Remarkably, the effect of DGAT inhibitors was similar in WT and seipin knockout cells
374 (Figure 5F-G). This indicates that the deficiency of seipin brought no additional impact to CE
375 LD formation beyond what was achieved by inhibition of TG synthesis alone. Taken together,
376 these results suggest that seipin affects CE LD formation via TGs: TGs cluster more readily
377 than CEs in the bilayer and associate with seipin, which in turn helps to co-cluster CEs and
378 promote CE LD formation.

379

380 **Discussion**

381 CEs form metastable supercooled liquids and at high CE concentrations, supercooled smectic
382 liquid crystalline droplets. They tend to crystallize, even at body temperature. Such
383 crystallization could be relevant to cholesterol-related disorders such as atherosclerosis,
384 fatty liver disease, and Niemann-Pick type C disease (40, 41). Yet, it seems that cells manage
385 to efficiently store CEs in metastable liquid forms in LDs, and prevent crystal formation.

386

387 At 37°C, the liquid crystalline phase appears in droplets when the CE-to-TG ratio exceeds
388 9/1, a threshold that decreases with temperature (Figure 1F)(32). In this regime, CE
389 molecules are highly ordered and form radial lamellar phases(32–34), likely arising from CE-
390 CE dimerization capacity (Figure 3). In the ER bilayer, these characteristics of CE molecules
391 would reduce the freedom of CEs and possibly retard their arrangement to condense in a
392 droplet. This would explain the delayed nucleation of CE droplets in model membranes, at
393 10-15% (CE/phospholipids) compared with 3-4% for TG. This implies that if CEs are not
394 removed from the ER membrane they would reach a high concentration, which could be
395 deleterious to the membrane.

396

397 Given the particularity of the crystalline phase, CEs should require specific membrane
398 settings for condensing into LDs. We found that TG, and not diacylglycerol, for example,
399 fulfills this need by acting as a catalyst, precipitating the incorporation of CEs into nascent
400 LDs. Indeed, with its lower nucleation concentration, TGs can form clusters that more
401 favorably incorporate CE molecules than CE clusters do. We speculate that the critical TG
402 concentration needed to catalyze CE LD nucleation is the one preventing the formation of a
403 crystalline phase in the ER, i.e. 1/9 of TG/CE at 37°C. Afterward, a nucleated CE-rich LD grows
404 by acquiring more CE molecules and thereupon enters the liquid crystalline regime.

405

406 As the prime TG-clustering factor(8, 10, 13, 14), seipin can control CE LD formation sites, as
407 long as TG molecules are available in the ER. Indeed, by interacting with TGs, seipin forms TG
408 pre-clusters that can recruit CEs to nucleate CE LDs. Accordingly, the inhibition of DGATs
409 severely decreased the number of nucleation events, as fewer TGs would be available. In
410 agreement with this model, in *Saccharomyces cerevisiae*, CE LDs form at seipins but, when
411 TG accumulation is completely inhibited by the removal of Lro1 and Dga1, the sole enzymes

412 making TGs, few LDs form away from seipins(28). Similarly, retinyl palmitate or squalene is
413 better incorporated into LDs when TG synthesis is ongoing(26, 28).

414

415 Since the accumulation of neutral lipids would be deleterious to the ER membrane(27) and
416 hence, overall cell functioning, removing them rapidly via LD formation is critical. Based on
417 cell observations, *in vitro*, and *in silico* data, TG seems to be so far the neutral lipid with the
418 lowest nucleation concentration; it is rapidly storable in LDs, possibly reflecting why it is the
419 most ubiquitous neutral lipid across many living systems. Therefore, accompanying TG
420 synthesis with the synthesis of other neutral lipids of lower nucleation efficiency might
421 represent a strategy for cells to ensure efficient nucleation of the latter. At seipins where
422 TGs are pre-clustered, these neutral lipids would better interact with TGs and nucleated LDs:
423 the initial TG seeds will act as thermodynamic pumps, retrieving other neutral lipids from the
424 bilayer. In this view, the role of seipin is coupled to TG, as seen in yeast(28). This proffers an
425 underappreciated role to TGs in LD formation, beyond their traditional energy storage
426 function.

427

428 Based on our data, mammalian cells must keep a background of TG production in the ER
429 membrane during lipogenic periods, even if TGs were not the primary neutral lipid stored.
430 This TG seed would play a critical role in nucleating LDs enriched in the synthesized neutral
431 lipid, at seipins. Such a surveillance role was well illustrated when the cells were supplied
432 with a tiny amount of oleic acid, to generate TGs under conditions of high cholesterol
433 feeding. There, many more CE-rich LDs were made as compared with no oleic acid-fed cells.
434 The proposed function of TG on CE LD assembly likely depends on the cell physiology and
435 may be critical for cell types that may have more difficulty in handling CE storage. For
436 instance, Cos7 cells strongly relied on TG synthesis to solubilize CEs, as they were more
437 sensitive to DGAT inhibition and barely assembled CE LDs under this condition
438 (Supplementary Figure 2J-O). In agreement with this, Cos7 cells did not display any liquid
439 crystalline organization in CE LDs upon cholesterol feeding, as opposed to HeLa cells
440 (Supplementary Figure 2P-R).

441

442 Finally, cells may systematically coordinate the level of TG synthesis when other neutral
443 lipids are made. Such a strategy would enable them to rapidly remove the latter from the ER

444 bilayer via the capacity of TG, better than other neutral lipids, to efficiently cluster and form
445 droplets. In this view, seipin would globally define the formation sites of LDs via clustering
446 TG.
447

448 **Materials and methods**

449 **Materials**

450 DAPI (Sigma, D9542), LD540 (Princeton BioMolecular Research), Autodot (Abgent,
451 SM1000b), DGAT1 inhibitor (Sigma PZ0207), DGAT2 inhibitor (SigmaPZ0233). Cell culture
452 reagents and general reagents were purchased from GibCo/Thermo Fisher, Lonza, and
453 Sigma-Aldrich. Lipoprotein-deficient serum (LPDS) was made from fetal bovine serum (FBS)
454 as previously described (42)). Methyl- β -cyclodextrin for cell culture (#C4555) was purchased
455 from Sigma Aldrich, cholesterol (Avanti, 70000P) was purchased from Avanti.

456 The cholesterol/methyl- β -cyclodextrin solution was prepared at a final concentration of
457 1mM of cholesterol as following. A suitable amount of methyl- β -cyclodextrin was dissolved
458 in cell culture media and then incubated with crystal cholesterol at 1/20 molar ratio
459 (cholesterol/ methyl- β -cyclodextrin) for 24 h with agitation at 37°C. The resulting solution
460 was filtered using 0.2 μ m syringe filter and conserved at 4°C until use.

461 For 1mM of oleic acid containing media preparation, 10% of BSA solution in DPBS (#A1595
462 Sigma Aldrich) was mixed with cell culture media at 1/10 v/v ratio. The resulting solution was
463 incubated with pure oleic acid (#O1383 Sigma Aldrich) to obtain a final concentration of 1
464 mM. The resulting mixture was vortexed then sonicated for a few minutes and incubated at
465 37°C for one hour. The resulting solution was filtered using 0.2 μ m syringe filter and
466 conserved at 4°C until use.

467

468 **Cell culture and lipid manipulations**

469 A431 cells (ATCC, Cat# CRL-1555, RRID:CVCL_0037) were maintained in Dulbecco's Modified
470 Eagle's Medium (DMEM) supplemented with 10 % FBS, penicillin/streptomycin (100 U/mL
471 each), and L-glutamine (2 mM) at 37 °C in 5% CO₂. All cell lines were regularly tested
472 negative for mycoplasma infection by PCR. For lipid droplet imaging experiments cells were
473 delipidated by 3 to 4- day treatment with serum-free medium supplemented with 5% LPDS.
474 For a more stringent delipidation cells were treated with DGAT 1&2 inhibitors (5 μ M each)
475 for the final 18 h of delipidation where indicated. DGAT_i indicates treatment with both
476 DGAT1 and DGAT2 inhibitors. Following delipidation cells were loaded with methyl-beta-
477 cyclodextrin complexed cholesterol in 5% LPDS for indicated concentrations and times or
478 with oleic acid in complex with BSA in 8:1 molar ratio prepared as described (43) in 5% LPDS.
479 For 24 h lipid loading experiments, COS7, HeLa and A431 cells were maintained in High
480 Glucose with stabilized Glutamine and with Sodium Pyruvate Dulbecco's modified Eagle's
481 Medium (DMEM) (Dutscher) supplemented with 10% fetal bovine serum and 1%
482 penicillin/streptomycin at 37°C and 5% CO₂. Cells were plated in Mattek dishes (#P35G-0-20-
483 C) for 24 h, then incubated with the culture media containing cholesterol, oleic acid or both
484 of them at the indicated concentration and time. When mentioned, DGAT1 and DGAT2
485 inhibitors were added at 5 μ M during lipid treatment.

486

487 **Stable cell lines**

488 A431 Seipin-KO (SKO) cells were generated with CRISPR/Cas9 technology as described (23).
489 The SKO clone in this study corresponds to S2AB-15 in (23). Generation of endogenously
490 tagged seipin-GFPx7 cells and generation of seipin-sfGFP control and seipin NE-trap cells are
491 described in (12). To generate end-seipin-GFPx7 cells, endogenous seipin was tagged with
492 GFP11x7 (Addgene #70224 (63), a gift from Bo Huang), which becomes fluorescent upon
493 self-complementation with a non-fluorescent GFP1-10 fragment expressed in the same cell
494 (63). We utilized a co-selection strategy for simultaneous tagging of endogenous seipin and

495 integration of the cassette overexpressing GFP1-10 into the AAVS1/Safe Harbor locus
496 through homology directed repair. Three plasmids (1: Seipin-GFP11x7 homology directed
497 repair template; 2: GFP1-10 overexpression cassette with puromycin selection marker on
498 AAVS1 integration template; 3: Cas9, sgBSCL2/seipin, sgAAVS1 overexpression plasmid) were
499 transfected at 5:1:4 ratio into A431 cells. Selection was done with puromycin and single
500 clones were isolated using limiting dilution and based on GFP fluorescence. GFP1-10
501 fragment was codon-optimized and synthesized by Genescript because the plasmid
502 (Addgene #70219 (63) a gift from Bo Huang) was of low codon adaption index (CAI, analyzed
503 at www.genscript.com/tools/rare-codon-analysis) and was poorly expressed by us in human
504 cells. Homozygous knock-in validated by genomic PCR and by western blot with an in-house-
505 generated antibody against seipin and an antibody against GFP.

506

507 **Cell stainings**

508 For confocal and widefield imaging of fixed cells, cells were washed with PBS and fixed with
509 4% PFA in 250 mM HEPES, pH 7.4, 100 μ M CaCl₂ and 100 μ M MgCl₂ for 20 minutes.
510 Subsequently, cells were washed with PBS, quenched in 50 mM NH₄Cl for 15 minutes and
511 washed with PBS. To stain lipid droplets and nuclei, cells were incubated for 30 minutes with
512 LD540 (1 μ g/mL) and DAPI (5 μ g/mL) in PBS at room temperature. After staining, cells were
513 washed with PBS and imaged in PBS. For live Airyscan imaging, cells were grown on a LabTek
514 II #1.5 glass-bottom dish coated with 10 μ g/mL fibronectin. Lipid droplets were stained with
515 Autodot (0,1 mM) for 5 min and imaged in Gibco FluoroBrite DMEM supplemented with 5%
516 LPDS at +37 °C, 5% CO₂.

517

518 **Imaging and image analysis**

519 For lipid droplet analysis cells were delipidated and cultured on 384- or 96-well high content
520 imaging plates (Corning) and imaged with PerkinElmer Opera Phenix automatic spinning-disk
521 confocal microscope using 63 x water objective, NA 1.15. Lipid droplets and seipin-sfGFP in
522 control and NE-trap cells were imaged with Nikon Eclipse Ti-E inverted widefield
523 fluorescence microscope using a 40 x air objective, NA 0.75, and 1.5 zoom. Z-stacks were
524 acquired to span whole cells on LD540 and DAPI channels. Image stacks were deconvolved
525 with Huygens batch processing application (<https://svi.nl/HuygensSoftware>, v. 22.10) and
526 image stacks were maximum intensity projected with custom MATLAB scripts
527 (<http://www.mathworks.com/products/matlab/>, v. 9.2.0.538062; scripts are available upon
528 request). Lipid droplets were detected with Ilastik (v. 1.3.2) (44) by pixel and object
529 classification utilizing machine learning algorithms and final binary images were used for
530 analysis in CellProfiler (45). Automatic cell segmentation and automatic image analysis were
531 done with CellProfiler with a previously described protocol (12, 46, 47). Cell nuclei were
532 detected with Otsu adaptive thresholding method in DAPI images and the cytoplasm was
533 detected as the faint background on the DAPI channel. All high-content imaging experiments
534 were carried out 2-3 times with similar results, with hundreds of cells analyzed per condition
535 and reported as described (12, 14, 48). For Airyscan images cells were imaged with Zeiss LSM
536 880 confocal microscope equipped with Airyscan detector using a 63 x Plan-Apochromat oil
537 objective, NA 1.4. Images were Airyscan-processed automatically with Zeiss Zen software (v.
538 2.3). Fiji (v. 1.51), CorelDraw (v. 24.1.0.360), and GraphPad Prism (v. 7.04) were used for data
539 visualization.

540

541 **Simulation System**

542 Seipin oligomer (transmembrane helices + luminal domain; or seipin luminal domain only)
543 were taken from our previous work (14). Membrane systems with varying concentrations of
544 CE (cholesteryl oleate) and TG (triolein) were prepared using the protocol described in our
545 previous work (14). For seipin simulations, the oligomer was inserted into the membrane
546 systems (see Table 1) using the protocol described in our previous work (14). In all systems,
547 unless specified, the neutral lipids (CE and TG) were randomly distributed around the seipin
548 oligomer. The resulting systems were solvated and neutralized using counter ions. 0.15 M
549 KCL ions were added to mimic the physiological salt concentration in the cytosol.
550

551 **Simulation protocols**

552 Simulations were performed using Gromacs simulation package ver 2019.6 (48).
553 Charmm36/m forcefield with virtual interaction sites (49–52) was used for the proteins,
554 lipids, water, and counter ions. This was done to enable using a larger integration time step
555 of 4 fs. TIP3P water model was used. The systems were energy minimized, pre-equilibrated
556 under constant pressure and temperature for 1 ns. Nose-Hoover thermostat (53) was used
557 to maintain the temperature at 310 K with a coupling constant of 0.5 ps while pressure was
558 maintained at 1 atm using the Parrinello-Rahman barostat (54) with a coupling constant of 2
559 ps. Verlet cutoff scheme was used to update the neighbor list every 10 steps. Covalently
560 bonded hydrogen bonds were constrained using LINCS algorithm (55). A value of 1.0 nm was
561 used to cut-off short-range electrostatic and van der Waals interaction while Particle Mesh
562 Ewald method (56) was used to treat long range electrostatic interactions. Production
563 simulations of μ s timescales were performed (see Table 1).
564

565 **Methods for free energy calculations**

566 To investigate the energetics and the mechanism of triglyceride (TG) and cholesteryl ester
567 (CE) binding to seipin, we performed two separate sets of free energy computations using
568 the umbrella sampling method. To simplify the systems for the free energy calculations, we
569 used a seipin monomer instead of the seipin protomer. Therefore, TG and CE bound seipin
570 monomers isolated from our previous simulations (14) and current study respectively, were
571 first re-embedded in a sufficiently large 100 mol% POPC membrane (using a hexagonal prism
572 of size 145 nm X 120 nm). These simplified membrane systems were prepared using the
573 CHARMM-GUI (56, 57). An integration time step of 4 fs was used for the simulations by
574 employing the Hydrogen Mass Repartitioning method (57, 58). After a series of short
575 equilibration simulations, the resulting configurations were used to start pulling simulations,
576 in which both TG and CE were pulled away from the protein in two separate 100 ns
577 simulations at a rate of 0.00005 nm/ps using a spring constant of 10000 kJ/mol/nm². The
578 lateral distance between the center of mass of the CA atoms of S165 and S166 and the polar
579 head group atoms for each lipid was used as the reaction coordinates for both pulling and
580 subsequent umbrella sampling simulations. The initial configurations for the umbrella
581 sampling windows were chosen from the pulling simulations at a 0.1 nm interval. A total of
582 about 50 windows were simulated spanning a range of 0.3-5.2 nm along the reaction
583 coordinate. Each window was simulated for 100 ns and a uniform force constant of 1000
584 kJ/mol/nm² was used in each window. The simulation results were unbiased and the free
585 energy profiles were generated using the Weighted Histogram Analysis Method (59).
586

587 **Analysis of simulation data**

588 Standard GROMACS tools were used for analysis. VMD (60) was used for visualizing the
589 trajectories and for rendering images. To calculate the fraction of CEs clustered in the
590 bilayer, we calculated the smallest distance between a chosen CE and the rest of the CE
591 molecules. A distance of 0.35 nm between any two CE molecules was chosen as a criterion
592 to consider the interaction between them. A cluster refers to the interaction between any
593 two molecules. Using the same criteria, we also calculated interaction and cluster formation
594 between CE and TG in mixed neutral lipid simulations. The values were calculated over the
595 entire simulation period and averaged over simulation sets for each system.

596

597 **Phase transitions and emulsion experiments**

598 In the phase transitions and emulsion experiments, cholesteryl oleate (CE, Sigma-Aldrich)
599 was heated to 37°C and 50°C using hot baths. Emulsion experiments were performed by
600 vortexing for 10 s and sonicating for 10 s, 5 μ L of previously liquefied CE (or mix between CE
601 and Triolein (TG, Sigma-Aldrich)) in 70 μ L of 50°C hot HKM buffer (50 mM HEPES, 120 mM
602 Kacetate, and 1 mM MgCl₂ (in Milli-Q water) at pH 7.4 and 275 \pm 15 mOsm). Silicone oil
603 emulsions were made using the same protocol with the HKM buffer replaced by Silicone oil
604 (100 cst, Sigma-Aldrich). Emulsions were then imaged using polarized confocal microscopy.

605

606 **Crystallization experiments**

607 The crystallization experiments were conducted by using a spectrophotometer cuvette as a
608 chamber. The droplets of the emulsion would then go up and flatten at the water-air
609 interface. Video were recorded using a u-eye fast imaging camera on a bresser optical
610 microscope.

611

612 **Cholesteryl oleate solubility experiments**

613 In vitro solubility experiments were performed by mixing CE and TO, and CE and
614 Diacylglycerol (DAG, Sigma Aldrich) in Eppendorfs. Pictures of the mixtures at different CE
615 concentrations were then analyzed using ImageJ to measure the opacity.

616

617 **Imaging of lipid droplets and image analysis using polarized light**

618 Cells experiments were imaged live at room temperature or 37°C, when indicated, with an
619 60x objective on a Zeiss LSM800 microscope. Images were then first analyzed using the
620 segmentation “WEKA” plugin in Fiji. The algorithm was trained for each set of experiments
621 and was error-checked by hand on test samples. WEKA gave a black-and-white segmentation
622 of the images. Images were then treated with watershed and despeckle before using the
623 “Analyze particles” plugin to determine the number and size of LDs.

624

625 **GUV and DEV preparation**

626 Giant Unilamellar Vesicles (GUVs) were composed of 69 mol% DOPC, 30 mol% DOPE (Avanti
627 polar lipids, Inc), 0.5 mol% Rhodamine-DOPE. For the nucleation experiments, CE/ CE-bpy
628 (18:2 TopFluor[®] cholesterol, Sigma-Aldrich) (99,5:0,5) GUVs were prepared by electro-
629 formation at 35°C and imaged at room temperature. PLs and mixtures thereof in chloroform
630 at 2.5 mM were dried on an indium tin oxide (ITO) coated glass plate. The lipid film was
631 dessicated for 1 h. The chamber was sealed with another ITO coated glass plate. The lipids
632 were then rehydrated with a sucrose solution (275 \pm 15 mOsm). Electro-formation was
633 performed using 100 Hz AC voltage at 1.4 Vrms and maintained for at least 2 h. This low
634 voltage was used to avoid hydrolysis of water and dissolution of the titanium ions on the

635 glass plate. GUVs were directly collected with a Pasteur pipette. CE artificial LDs (aLDs) were
636 prepared as explained above. To make DEVs, GUVs were incubated with the aLDs for 10 min.
637 The GUV-aLDs mixture was then placed on a glass coverslip pretreated with 10% (w/w) BSA
638 and washed three times with buffer. Repeated experiments were conducted without
639 treating the glass coverslip in order to burst the DEVs flat, and similar results were observed.

640

641 **FRAP experiments**

642 FRAP experiments were performed on CE/CE-bpy-containing GUVs that had previously been
643 deposited on untreated coverslips. The fluorescence recovery of CE-bpy signal is normalized
644 by the fluorescence signal before bleaching. Images were acquired every second during the
645 course of the experiment, with a Zeiss LSM 800 confocal microscope.

646

647 **Droplet/membrane partitioning analysis**

648 The fluorescence signal of the CE-bpy was quantified in the bilayer and in the droplet, by
649 fitting a gaussian curve on a line profile in Fiji. The ratio of the maxima in the droplet and in
650 the bilayer is used as an output. In the case of burst DEVs, the fluorescence signal was
651 averaged over a surface. The data have been normalized between the experiments in order
652 to compare the droplet/membrane ratios between the different oils used.

653

654 **Thin layer chromatography**

655 Cells were starved in 5% LPDS for 4 days + DGATi for the last 18 h, collected in 2 % NaCl and
656 lipids were extracted as described (61). Equal amounts of each sample based on protein
657 concentration were separated by thin layer chromatography (TLC) in hexane: diethyl ether:
658 acetic acid (80:20:1) as the running solvent. CE amounts were quantified from charred TLC
659 plates using ImageJ.

660

661 For 24h loading conditions, indicated cells were seeded at 70% of confluence and incubated
662 overnight. Cells were treated as indicated in the legend. Treatment cell culture media was
663 removed and the cells were washed three time with DPBS then Trypsine-EDTA (Gibco™
664 #15400054) was added to detached the cells. Cells were recovered by the normal cell culture
665 media and centrifuged. Media was discarded and the cells pellet was resuspended in DPBS
666 then centrifuged and resuspended again in DPBS. Cells were counted in each condition and
667 the volume was adjusted to get the same concentration of cells in each sample. 200 µl of
668 cells suspension was added to 4 ml CHCl₃ /methanol (2:1, v/v) then mixed by vortexing. 800
669 µl of water were added and mixed by vortexing. Samples were allowed to sit at room
670 temperature for 1 h. Samples were vortexed again and sat until the separation of aqueous
671 and organic phases. Aqueous phase was aspirated and the organic phase was dried under
672 stream of nitrogen. Lipids were resuspended in 50 µl of CHCl₃ /methanol (2:1). Glass
673 precoated silica-gel 60 plates (Merck, Darmstadt, Germany, 20 x 20 cm) were used for
674 neutral lipids separation. The separation was carried out with n-hexane/diethyl ether/glacial
675 acetic acid (70:30:1, v/v/v, SIGMA-Aldrich) in an all-glass chromatography chamber at room
676 temperature. The plates were then dried for 30 min in a fumehood. The plates were
677 revealed by spraying with 50% aqueous sulphuric acid (VWR) (H₂SO₄:H₂O 1:1, v/v),
678 overnight dring and heating in the oven at T=115°C for 30 min. The identification of CE and
679 TG was made by dissolving each standard compound in CHCl₃/Methanol (2:1, v/v). CE and
680 TG amounts were quantified using ImageJ.

681

682 **Data availability**

683 Data supporting the findings of this manuscript are available from the corresponding author
684 upon request. A reporting summary for this article is available as a Supplementary
685 Information file. Source data are provided as a Source Data file.

686

687 The datasets generated during and/or analysed during the current study are in the source
688 data file and/or available from the corresponding author on reasonable request.

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862 **Figure captions.**

863 Figure 1. CE can form supercooled droplets in vitro

- 864 (A) **Top:** Schematic representation of bulk CE upon heating from 25°C to 50°C and cool-
865 ing down to 37°C. Bulk CE liquefied when heated above its melting point and solidi-
866 fied upon cooling to 37°C. **Bottom:** Schematic representation of CE emulsification
867 protocol. CE was heated above its melting point and HKM buffer was added at 50°C.
868 The mixture was emulsified by vortexing and sonicating, and then imaged under con-
869 focal microscopy. The droplets appeared to be (meta)stable and thus supercooled.
- 870 (B) CE and TG emulsions were imaged at the water-air interface in brightfield. **Left:**
871 Schematic representation of an oil droplet spreading at the water-air interface. **Mid-**
872 **dle:** Image of a CE droplet spreading at the water-air interface. Some droplets re-
873 vealed concentric rings. **Right:** Image of a TG droplet spreading. No concentric rings
874 were observed.
- 875 (C) Live imaging of the crystallization of a CE droplet upon meeting with a nucleation
876 seed.
- 877 (D) Supercooled CE droplets were imaged using polarized light microscopy on a tempera-
878 ture-controlled stage. **Left:** Image of a CE emulsion at 37°C. **Right:** Images of the
879 three polarized droplets phenotypes encountered.
- 880 (E) Analysis of the occurrences of the lipid droplets phenotypes, n = 236 droplets.
- 881 (F) Schematic segmentation of the CE/TG droplets' physical states according to the
882 CE/TG ratio and emulsion temperature. Droplets are stable and isotropic below 20%
883 of CE, metastable but isotropic until ~85% of CE near room temperature and exhibit a
884 liquid-crystalline phase above. Done at 0%, 20%, 50%, 85%, 95% and 100%.
- 885 (G) A431 cells imaged after 24 h of cholesterol (200 µM) or oleic Acid (40 µM) + choles-
886 terol (160 µM) feeding. Bodipy was added upon imaging for LD labelling and further
887 analysis (H). Experiments done in triplicates.
- 888 (H) Analysis of the fraction of LDs in LC form in (G), Mean +/- SD. n=66, 54 and 39 cells for
889 200 µM Cholesterol. And 54, 25, and 27 cells for 160 µM Cholesterol + 40 µM Oleic
890 Acid Each color point represents a data point from a replicate. The experiment was
891 independently repeated three times with similar results. **** p<0,0001 two-tailed
892 Nested t test.
- 893

894 Figure 2. TGs stimulate cholesterol lipid droplet assembly in cells.

- 895 (A) Maximum intensity projections of confocal images of lipid droplets. A431WT cells
896 starved in 5% LPDS for 4 days + DGATi for overnight and loaded with indicated con-
897 centrations of cholesterol/cyclodextrin for one hour +/- DGATi. Cells were fixed and
898 stained with LD540 and DAPI. Scale bar = 10 µm. The experiment was independently
899 repeated three times with similar results.
- 900 (B) Quantification of lipid droplet numbers per cell. n= 899 cells for 0 µM, 944 cells for 0
901 µM + DGATi, 734 cells for 50 µM, 1016 cells for 50 µM + DGATi, 547 cells for 100 µM,
902 948 cells for 100 µM + DGATi, 705 cells for 200 µM, and 864 cells for 200 µM + DGATi
903 condition. Mean +/- SEM. **** p< 0.0001, two-tailed student's t-test.

- 904 (C) A431WT cells loaded with cholesterol, oleic acid, or both cholesterol and oleic acid
 905 with indicated concentrations for 30 min +/- DGATi after similar starvation as in (A).
 906 Quantification of lipid droplet numbers per cell from confocal images of fixed cells
 907 stained with LD540 and DAPI. n= 631 cells for LPDS, 899 cells for 10 μ M OA, 1175
 908 cells for 20 μ M OA, 951 cells for 100 μ M chol, 1471 cells for 100 μ M chol + 10 μ M OA,
 909 929 cells for 100 μ M chol + 20 μ M OA, 1051 cells for 100 μ M chol + 10 μ M OA +
 910 DGATi, and 870 cells for 100 μ M chol + 20 μ M OA + DGATi condition. Mean+/- SEM.
 911 In 20 μ M OA + 100 μ M chol + DGATi, more CEs are synthesized compared to 10 μ M
 912 OA + 100 μ M chol + DGATi (see Supplementary Figure 2F), in line with more LDs be-
 913 ing formed. **** p< 0.001, two-tailed student's t-test.
- 914 (D) A431 cells imaged after 24 h of cholesterol (200 μ M) or cholesterol (200 μ M) and
 915 DGAT inhibitors feeding. Bodipy was added upon imaging for LD labelling and analysis
 916 (F).
- 917 (E) A431 cells imaged after 24 h of cholesterol (190 μ M) + oleic Acid (10 μ M) or choles-
 918 terol (190 μ M) + oleic acid (10 μ M) and DGAT inhibitors feeding. Bodipy was added
 919 upon imaging for LD labelling and analysis (G).
- 920 (F) Analysis of (D) and (E). Fraction of liquid crystalline LDs, Mean +/- SD. N=65, 54 and
 921 39 cells for 200 μ M Cholesterol. N=49, 42, and 37 cells for 200 μ M Cholesterol +
 922 DGATi. N=40, 20 and 34 cells for the 190 μ M Cholesterol + 10 μ M OA. N=41,27, and
 923 26 for 190 μ M Cholesterol + 10 μ M OA + DGATi. Each color point represents a data
 924 point from a replicate. The experiment was independently repeated three times with
 925 similar results. ns p=0.4647 & p=0.9444, no significant differences two-tailed Nested t
 926 test.
- 927 (G) Analysis of (D) and (E). Number of LDs per cell, Mean +/- SD. N=65, 54 and 39 cells for
 928 200 μ M Cholesterol. N=49, 42, and 37 cells for 200 μ M Cholesterol + DGATi. N=40, 20
 929 and 34 cells for the 190 μ M Cholesterol + 10 μ M OA. N=41,27, and 26 for 190 μ M
 930 Cholesterol + 10 μ M OA + DGATi. Each color point represents a data point from a rep-
 931 licate. The experiment was independently repeated three times with similar results.
 932 (Left to Right) * p=0,027, **p=0,0065, ns p=0,0547, ** p=0,0047 two-tailed Nested t
 933 tests.

934 Figure 3. CEs condense into droplets in model membranes.

- 935 (A) GUVs were made with different concentrations of CE and imaged under confocal mi-
 936 croscopy. **Top:** Images of gradually CE-concentrated GUVs. CE condensates started
 937 appearing from the concentration of 15 mol%. **Bottom:** Schematic representation of
 938 gradually increasing CE concentration within GUV bilayer.
- 939 (B) **Left:** Schematic representation of GUV splashing on a bare glass coverslip. **Right:** Mi-
 940 croscope image of a splashed GUV, appearing flat on the glass surface. CE conden-
 941 sates were still visible and immobilized.
- 942 (C) Signal recovery in a totally bleached CE condensate from splashed GUV as shown in
 943 (C). The signal was normalized by the initial fluorescence signal.
- 944 (D) Analysis of (C). Quantification of the amount of free CE in the membrane by measur-
 945 ing the CE/PL fluorescence ratio in splashed GUVs as shown in (C). Points: Mean \pm SD,
 946 5-10 GUVs per experimental point.

- 947 (E) Analysis of (C). Quantification of the occurrences of droplet-containing GUVs accord-
 948 ing to the [CE/PL] ratio. Above the [CE/PL] ratio of 15%, both the CE enrichment in
 949 the membrane and the occurrences of droplet containing GUVs appear to be almost
 950 constant, suggesting that the critical concentration is around 15%. N=9-37 GUVs per
 951 concentration.
- 952 (F) Representation of CE distribution in a model ER bilayer at 5 mol%, 10 mol%, and 15
 953 mol% CE at the end ($t=2 \mu\text{s}$) of the simulation period. Top-view (left) and side-view
 954 (right) representation of the system. CE molecules are shown in orange. Phosphate
 955 atoms of the membrane lipids are shown in tan in the side-view representation. Oth-
 956 er components of the system (membrane lipids, water, and ions) are not shown for
 957 clarity.
- 958 (G) Plot showing the fraction of CE molecules clustered in the bilayers during the simula-
 959 tion. The remaining CE molecules that are not bound to clusters are individual mon-
 960 omers. The data have been averaged over three simulation repeats.

961

962 Figure 4. TGs facilitate CE clustering and incorporation into droplets.

- 963 (A) **Left:** Schematic representation of Droplet Embedded Vesicles (DEVs) protocol. An
 964 emulsion of either CE, TG, DAG or SQ was mixed with CE-containing (10%) PC/PE
 965 (70/30) GUVs. The droplets go in between the two layers of the GUVs, forming a DEV.
 966 The CE already present in the membrane can then diffuse to the newly embedded
 967 droplet. **Right:** Representation of the droplet/membrane partitioning ratio. The CE-
 968 bodipy fluorescence in the embedded droplet was compared to the CE-bodipy fluo-
 969 rescence in the membrane. The ratio indicates how much the CE-bodipy diffused
 970 from the membrane to the droplet as explained in (G).
- 971 (B) Quantification of (A) for TG, DAG, and SQ DEVs. Mean \pm SD. 5, 9, and 5 DEVs for TG,
 972 6, 10, and 8 for DAG, and 5, 7, and 8 for SQ conditions. Each color point represents a
 973 data point from a replicate. The experiment was independently repeated three times
 974 with similar results. (Left to Right) $**p=0,0044$, $****p<0,0001$, ns $p=0,1145$, two-
 975 tailed Nested t-tests.
- 976 (C) Representation of CE (orange) and TG (blue) distribution in a model ER bilayer at 2.5
 977 mol% of each neutral lipid; at TG-CE concentration of 3 mol%-4mol% respectively and
 978 at 5 mol% of each neutral lipid at the beginning ($t = 0$) and at the end ($t=2 \mu\text{s}$) of the
 979 simulation period. Top-view (left and center) and side-view (right) representation of
 980 the system. Phosphate atoms of the membrane lipids are shown in tan in the side-
 981 view representation. Other components of the system (membrane lipids, water, and
 982 ions) are not shown for clarity.
- 983 (D) Plot showing the fraction of CE molecules clustered in the bilayers during the simula-
 984 tions. The data have been averaged over three simulation repeats. Mean \pm SD.
- 985 (E) Plot showing the fraction of CE molecules interacting with CE molecules (CE-CE) and
 986 CE molecules interacting with TG molecules (CE-TG) in 3% TG + 4% CE system. The
 987 data have been averaged over three simulation repeats.

988

989 Figure 5. Seipin controls CE LD nucleation sites via its TG clustering capacity.

990 (A) Airyscan images of cells with endogenously GFPx7-tagged seipin starved for 3 days in
991 5% LPDS + DGATi for overnight, washed and loaded with 100 μ M cholesterol
992 cyclodextrin for 90 min, stained with Autodot to visualize lipid droplets and imaged
993 live. Arrowheads indicate a stable contact between seipin and a lipid droplet during 4
994 seconds. Scale bar = 1 μ m. The experiment was independently repeated three times
995 with similar results.

996 (B) Maximum intensity projections of deconvolved widefield images of seipin-sfGFP in
997 control and seipin NE-trap cells. (Left) Confocal images of lipid droplets in control and
998 seipin NE-trap cells. (Right) Cells were starved for 3 days in 5% LPDS + DGATi over-
999 night and loaded with 200 μ M cholesterol cyclodextrin + DGATi for 3 h, fixed and
1000 stained with DAPI and LD540. Scale bar = 10 μ m. LD area overlapping with nuclei
1001 quantified as fraction of LDs area at NE. Control n= 727 and NE-trap n=671 cells.
1002 Mean+ SD and all individual data points. **** p< 0.0001, two-tailed student's t-test.
1003 An independent similar experiment presented in Supplementary Figure 5A.

1004 (C) Top-view representation of CE molecules (5 mol%, shown in orange) around the
1005 seipin oligomer associated with a model ER membrane. **Left:** At the beginning (t = 0).
1006 **Middle:** At the end (t = 3 μ s) of the simulation period (zoomed in to show CE associa-
1007 tion with α 2- α 3 helices). Each protomer is shown with a different color. **Right:** A
1008 close-up view showing the interaction of a CE molecule with the residues S165 and
1009 S166 on a seipin protomer. Yellow bars represent the interacting atoms between the
1010 residues and CE.

1011 (D) Minimum distance between a tagged CE molecule and the residues S165 and S166 of
1012 a single protomer.

1013 (E) **Left:** Following the S165A-S166A mutation, the minimum distance between the
1014 tagged CE molecule (previously bound to S166) and the residues A165 and A166 of
1015 the protomer in which the CE was bound. The data have been averaged over three
1016 simulation repeats.

1017 **Right:** Following the S166A mutation, the minimum distance between the tagged CE
1018 molecule (previously bound to S166) and the residues S165 and A166 of the
1019 protomer in which the tagged CE was bound. The data have been averaged over
1020 three simulation repeats.

1021 (F) Maximum intensity projections of confocal images of Seipin-KO (SKO) cells starved in
1022 5% LPDS for 4 days + DGATi for overnight and loaded with indicated concentrations
1023 of cholesterol cyclodextrin for one hour +/- DGATi. Cells were fixed and stained with
1024 LD540 and DAPI. Scale bar = 10 μ m.

1025 (G) Quantification of lipid droplet number per cell. n= 905 cells for SKO LPDS, 1013 cells
1026 for SKO LPDS + DGATi, 946 cells for SKO 50 μ M, 1084 cells for SKO 50 μ M chol +
1027 DGATi, 918 cells for SKO 100 μ M, 1009 cells for SKO 100 μ M + DGATi, 1048 cells for
1028 SKO 200 μ M, and 895 cells for SKO 200 μ M + DGATi condition. Mean +/- SEM, includ-
1029 ing data from (2B).

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1049

1050 **Author contribution**

1051 A.R.T., E.I. and I.V. designed the research. C.D. conducted all in vitro experiments and cell
1052 experiments using polarized light, with the help of M.C., M.O. and A.B. L.V. conducted all cell
1053 experiments focusing on early LD formation, with the help of V.T.S. X.P. performed simulations,
1054 except for the energy calculation of seipin interaction with neutral lipids carried out by G.E. All
1055 authors analyzed the data. A.R.T. and E.I. wrote the manuscript, reviewed and edited by all co-
1056 authors.

1057

1058 **Declaration of interests**

1059 The authors declare no competing interests.

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