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1 **Lipid droplet nucleation**

2

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25 **Abstract**

26 All living organisms can make lipid droplets (LDs), intracellular oil-in-water droplets,
27 surrounded by a phospholipid and protein monolayer. LDs are at the nexus of cellular lipid
28 metabolism and function in diverse biological processes. During the past decade,
29 multidisciplinary approaches have shed light on LD assembly steps from the endoplasmic
30 reticulum (ER): nucleation, growth, budding, and formation of a separate organelle. The
31 molecular mechanisms underpinning these steps are, however, still elusive. This review
32 focuses on the nucleation step, defining where and how LD assembly is initiated. We will
33 present how membrane biophysical and physicochemical properties control this step and
34 how proteins act on it to orchestrate LD biogenesis.

35

36

37

38 **Keywords.**

39 Lipid droplet biogenesis, phase transitions, seipin, membrane curvature, neutral lipids,
40 membrane chemistry

41

42

43

44 **Synopsis of lipid droplet biogenesis.**

45 Cells respond to excess nutrients or diverse stress stimuli by fabricating neutral lipids that
46 are subsequently packaged into LDs [1,2]. LDs, also called oil or lipid bodies [3], exert
47 several biological functions beyond metabolism [2,4]. How neutral lipids are fabricated is
48 rather well-known but how they are packaged into LDs remains more elusive [5–7]. Recent
49 studies have revealed biophysical and physicochemical factors assisting in the initiation of
50 LD assembly [8–12]. This review discusses how these factors impact LD nucleation and how
51 proteins may modulate them to control LD assembly. We focus on the thermodynamics of
52 phase transitions underlying LD nucleation and describe how this process is influenced by
53 membrane proteins, lipids, and mechanical properties [13].

54

55 **Neutral lipid synthesis.**

56 LD formation is initiated by the synthesis of **neutral lipids** (NLs) (see Glossary), which in
57 most eukaryotes takes place in the ER bilayer [5,14] (Fig.1a). In plastids, NL synthesis may
58 take place at the inner envelope membrane, equivalent to the plasma membrane of
59 prokaryotes [15]. The most abundant NLs in eukaryotes are **triacylglycerols** (TGs) and
60 **sterol esters** (SE) (Fig.1b) and the major enzymes generating them in mammals are the
61 diacylglycerol O-acyltransferase 1 (DGAT1) and acyl-coenzyme A: cholesterol

62 acyltransferase 1/sterol O-acyltransferase 1 (ACAT1/SOAT1), belonging to the membrane-
63 bound O-acyl transferase superfamily. Their cryo-electron microscopy structures show
64 interesting similarities in the proposed catalytic mechanism [16–19]. They have an
65 intramembrane catalytic chamber to which there are entrances from the cytoplasmic and the
66 lateral, intramembrane side (Fig.1a). The acyl-CoA enters via the cytoplasmic tunnel and
67 diacylglycerol (for DGAT1) or cholesterol (for ACAT1) may enter through the lateral,
68 transmembrane side (Fig.1a). The reaction product, TG, leaves laterally, i.e. inside the
69 bilayer, and this may also be the case for SE [16–18]. The synthesized NLs mix with
70 phospholipids (PLs) and are diffusible in the ER bilayer [11].

71
72 These enzymes have isoforms, namely DGAT2 and ACAT2, that also catalyze the
73 esterification reaction generating NLs, although DGAT2 is evolutionarily unrelated to DGAT1.
74 The structures of these isoforms are not yet resolved but they may mediate the synthesis of
75 specific NL pools under precise physiological stimulations [20–22]. ACAT2 is predominantly
76 expressed in enterocytes and hepatocytes and esterifies dietary cholesterol [23]. DGAT2
77 localizes around LDs, probably mediating LD-ER contacts [24], and may synthesize and
78 incorporate TG directly into LDs. DGAT2 is also capable of esterifying ceramides into
79 acylceramides to be stored in LDs [25]. Hence, DGATs and ACATs may esterify other
80 hydrocarbons with alcohol functions to generate NLs other than TG and SE.

81
82 ***The assembly steps of lipid droplets.***
83 How are NLs diffusible in the ER bilayer assembled into LDs? *In vitro*, when the
84 concentration of NLs reaches a critical level, a spontaneous condensation of NLs by phase
85 separation can occur, leading to the formation of an oil blister within the hydrophobic bilayer
86 region [11,26–28]: this first step of LD assembly is termed **nucleation** (Box1). Spontaneous
87 nucleation is also likely to happen in cells.

88
89 The second step is *growth*: following nucleation principles, the oil blister has to grow, by
90 adsorbing more NLs to become an LD or to resorb [13,29,30]. If many LDs are nucleated in
91 parallel, it is unfavorable for all of them to grow, unless they are stabilized against **Oswald**
92 **ripening** [13,31]. TGs diffuse in tubules roughly at the same rate as PLs, $\sim 4 \mu\text{m}^2/\text{sec}$ [11],
93 implying that a TG molecule can explore two LDs that are $2 \mu\text{m}$ apart in a second. If LDs are
94 not stabilized against ripening, the equilibrium state requires that only one LD, the larger one,
95 grows at the expense of the others that resorb [13,31].

96
97 The third step consists of the *budding* of a spherical LD into the cytosol [5,14]. This step
98 requires maintaining the PL and protein coverage of the cytosolic monolayer tighter than in

99 the luminal face [32]. Such asymmetry guarantees that the cytosolic leaflet remains of lower
100 **surface tension**, promoting LD emergence in this direction [33]. If proteins bind strongly to
101 emerging LDs from the ER lumen, LDs fail to fully emerge. This happens when lipidated
102 apolipoprotein B100 is overexpressed in the ER lumen of hepatocytes where it keeps LDs in
103 contact with the lumen [34].

104

105 The fourth step is likely a protein-assisted *stabilization of LD-ER contacts* or a potential
106 *detachment* of the LD from the ER bilayer. In either case, the budded LDs remain closely
107 tethered to the ER bilayer by several tethering proteins [35–38].

108

109 ***Thermodynamics of lipid droplet nucleation.***

110 We previously detailed the thermodynamics of phase transition in the context of LD
111 nucleation [13]. Briefly, NLs within the ER bilayer can spontaneously demix from the PLs and
112 condense into a lens when a critical nucleation concentration (CNC) is reached [39] (Fig.2a,
113 Figure I Box.2). The existence of a CNC is common to all phase separation phenomena
114 [13,29]; in the context of detergents, it is the critical micellar concentration. Nucleation is
115 associated with an energy barrier, $E_{\text{nucleation}}$, that needs to be overcome to generate stable NL
116 condensates (Figure I Box.2) [1]. The CNC for triacylglycerols (TG) in a flat 1-palmitoyl-2-
117 oleoyl-sn-glycero-3-phosphocholine bilayer is estimated as 3-5% (TG/PL) (Fig.2a) [26,28,40].
118 Below this threshold, TGs diffuse and form short-lived clusters, which size, lifetime, and
119 appearance frequency increase with the TG concentration [1]. Beyond the CNC, some TG
120 clusters can overcome $E_{\text{nucleation}}$ and transit into blisters or nascent LDs (Figure I Box.2) [1–4],
121 even though a residual fraction of TGs still mixes with the bilayer PLs [28,40,41] for entropic
122 reasons [1]. These nucleated LDs reduce the concentration of NLs in the bilayer to below the
123 CNC, and thereupon diminish the appearance frequency of new clusters [13,29]. In a uniform
124 protein-free bilayer, these steps happen stochastically (Fig.2a). In cells, the compositional
125 and structural heterogeneities of the ER membrane will induce local alterations of the energy
126 barrier and dictate where LDs nucleate [4,13] (Fig.2b,2c).

127

128 ***Role of membrane chemistry in lipid droplet nucleation.***

129 The chemical potential of NLs in a compartment represents the variation in free energy per
130 added NL molecule. The difference in chemical potentials between the condensed phase and
131 the dilute phase quantifies how favorable it is for NLs to stay in contact with the membrane
132 components as opposed to be into a nucleated LD [13]. The higher the chemical potential in
133 the bilayer, the less favorable it is for NLs to remain in contact with the membrane
134 components, and the higher the nucleation probability [13]. Therefore, the chemical potential
135 depends on the physicochemical properties of the membrane, which implies that the CNC

136 and $E_{\text{nucleation}}$ also depend on these physicochemical parameters, such as membrane proteins
137 and PLs [13] (Figure II Box.2). Changing the membrane PL composition indeed alters the
138 level of free TG within a bilayer [40,41] and, consequently, the nucleation efficiency of TG
139 blisters [41]. In particular, increasing dioleoylphosphatidyl ethanolamine, cholesterol, or
140 diacylglycerol (DAG), which are negatively curved lipids, promote the nucleation of TG
141 blisters [8,41]. Thus, the local enrichment of these lipids in the ER may help determine LD
142 nucleation sites. How exactly these lipids would promote LD nucleation is unclear [41] but
143 they may alter the bilayer physical properties, such as local curvature, **surface pressure** or
144 packing [42]. Generating PL packing defects exposes TGs to water, likely increases chemical
145 potential and favors TG condensation [11]. In any case, these observations support the idea
146 that local transient alterations in lipid composition can decrease the CNC and energy barrier
147 to promote nucleation. Conversely, changes in ER PL composition could also lead to
148 excessive accumulation of NLs in the ER membrane [8], and eventually to the nucleation of
149 abnormally large LDs.

150

151 ***The neutral lipid chemistry impacts nucleation.***

152 PLs and proteins are considered as major factors for LD assembly and NLs are often viewed
153 as “inert” apolar molecules with no impact on LD assembly. Instead, the NL chemistry
154 determines the interactions with the membrane constituents and sets the chemical potential.
155 TG and SE are the most abundant NLs in eukaryotes but many others exist in specific cells
156 from plants to animals [25,43,44] (Fig.1c-d). These diverse NLs are probably able to
157 spontaneously condense and nucleate LDs, as shown for example for phthiocerol
158 dimyocerosate [45]. Their contrasted chemistry proffers different chemical potentials, CNCs,
159 and nucleation energy barriers. Differences in chemical potential are partially reflected by
160 differential interactions with water, since the oil-water interfaces of NLs have different surface
161 tensions and propensities to recruit proteins [46]. The relevance of the NL chemistry for LD
162 nucleation is well illustrated by the capacity of TG to condense into droplets more efficiently
163 than SE in a DOPC bilayer [11], or by the abnormal accumulation of TG in the ER when DG
164 synthesis is inhibited [8]; squalene, which is a hydrocarbon and not an ester, tends to
165 accumulate in membranes and likely has a much higher CNC, resulting in fewer and larger
166 LDs [40,47]; likewise, retinol palmitate assembles into fewer and larger LDs than TG [48]. In
167 short, based on several *in vitro* characterizations [11,41,46,48,49], NLs differently interact
168 with membrane components (water, PLs, and proteins) and have different chemical
169 potentials. This suggests that specific ER membrane settings, e.g. in PL and protein
170 composition or curvature, may be required to trigger the condensation of a given NL into
171 LDs. Consequently, the NL composition would determine the nucleated LD phospholipidome
172 and proteome [4,46,48,50–55].

173

174 ***Lipid droplet nucleation at tubules vs. sheets.***

175 The ER membrane is made of sheets and tubules whose curvature affects protein and PL
176 sorting, coordinating biochemical reactions and vesicular trafficking [56–59]. TG and SE are
177 also differentially sorted by membrane curvature: relative to PLs, individual TG disfavor
178 tubules with increased curvature while SE is more enriched in them [11]. Other NLs can also
179 be expected to show differential enrichments in curved vs. flat membranes.

180

181 Curvature generates mechanical stresses [42,60], which might be enhanced by the presence
182 of NLs. Therefore, the prompted condensation of TG at tubules to nucleate an LD could be a
183 means to resorb bilayer stress. Another view of this scenario is that curvature increases the
184 chemical potential of TG. One aspect of curvature is to pull apart PLs, creating packing
185 defects or decreasing the bilayer surface pressure, hence promoting more contacts between
186 water and the hydrophobic bilayer core. This changes the interaction energy landscape of an
187 NL in the curved membrane. For TG, this interaction profile likely results in an increase in
188 chemical potential, since it preferentially moves to flatter regions or condenses [11]. In other
189 words, the CNC and the nucleation energy barrier for TG is smaller in tubules than in flat
190 membranes. As a result, the curvature is a catalyst for the assembly of TG LDs (Figure I-II
191 Box.2), and the frequency of nucleated LDs would follow the abundance of ER tubules [11]
192 (Fig.3a).

193

194 In contrast to TG, SE in tubules tends to stay as individual molecules diffusing faster than TG
195 [11]. Increasing the SE/TG ratio decreases the nucleation efficiency at tubules [11]. SE and
196 TG are both hydrophobic. If the spacing of PL by curvature were the sole reason for the
197 condensation of TG, to minimize contact with water, SE would behave similarly, but it does
198 not [11]. This suggests that other factors specifically impact SE and not TG. For example, SE
199 could decrease tubule stress when remaining as individual molecules, as opposed to TG.
200 Also, similarly to sterols [61], SE could alter the rigidity and surface pressure of the
201 membrane, to a direction unfavorable for nucleation (Box.2). Finally, relative to PLs, SE and
202 TG may have different orientations in the membrane and different interactions with
203 membrane components. These diverse options reflect the gap in our understanding of how
204 membrane curvature, chemistry, and mechanics impact the condensation of different NLs.

205

206 ***ER membrane shaping proteins regulate nucleation.***

207 The role of curvature in nucleation explains, at least partly, observations made on the impact
208 of ER shaping proteins on LD formation [10,12,62–67]. The dysfunction of several of such
209 proteins impedes the number of formed LDs, without necessarily impacting NL levels [12].

210 This is the case for example for the yeast peroxisomal membrane shaping protein Pex30, or
211 MCTP2 in mammals, [10,12] whose concomitant deletion with seipin generates large ER
212 structures low in curvature and abnormally filled with TG [12]. This is likely because the CNC
213 of TG (relatively to PLs) is high in these structures of zero curvature. Indeed, decreasing the
214 PL/TG ratio by inhibiting the PC synthesis enzyme PCTY1 may enable TG to reach the CNC,
215 and results in the recovery of LDs [12]. In this rescue experiment, fewer and larger LDs were
216 assembled compared to normal cells, as a signature of the higher CNC in flat membranes
217 (Fig.3a) [11]. Similarly to the PEX30/seipin deletion phenotype, when mammalian cells are
218 subjected to hypotonic medium, they make large flat ER structures bearing high amounts of
219 TG and are deficient in LDs [11].

220

221 Knowing the impact of membrane curvature on LD assembly, any ER shaping protein, as
222 recently shown for atlastin, Rabs, reeps, and torsins, or other ER structuring factors, such as
223 microtubules, septins, etc., will impact LD assembly [11,38,62–65,68,69]. The dysfunction of
224 several of these proteins is linked to hereditary spastic paraplegia phenotypes [2,64], but
225 whether the alteration in LD assembly is anecdotal or relevant for the disease pathogenesis,
226 remains to be elucidated. Finally, the alteration of LD formation by ER shaping proteins might
227 either be due to variations in the ER tubular fraction, promoting LD assembly, and/or to
228 altered NL/PL ratio, since the synthesis of NLs, like that of PLs [70], might happen at tubules.
229 These two contributions need to be untangled to better understand the impact of ER shaping
230 proteins on NL levels and LD nucleation.

231

232 The inner nuclear membrane (INM) possesses metabolic activity and can synthesize TG
233 [71,72]. This seems to be the case at least for the phospholipid: diacylglycerol
234 acyltransferase enzyme whose yeast homolog Lro1 has its catalytic activity in the nucleus
235 and mediates TG synthesis in the nucleus [71]. However, the generated NLs flow through the
236 interconnections between the INM and the ER and feed cytosolic LDs [71,73]. Why these
237 TGs do not directly assemble into nuclear LDs? An answer could be the lack of curvature at
238 the INM. The few nuclear LDs, particularly in hepatocytes [74,75], are mostly observed at
239 nuclear membrane invaginations [74] offering curvature. When seipin is artificially trapped
240 from the ER to the nuclear envelope with little curvature, LD assembly happens at the seipin
241 sites [31]. However, the careful analysis revealed that most of these LDs assembled close to
242 ER tubules adjacent to the nuclear envelope [11].

243

244 In short, the assembly of TG LDs is catalyzed by membrane curvature as otherwise, TG
245 simply fills membranes, incapable of undergoing efficient condensation [11]. Consequently,
246 the relative abundance of ER tubules may serve as a proxy for projecting on the capacity of a

247 cell type to handle excessive TG for storage. Cells with a relatively low abundance of ER
248 tubules may generate fewer and larger LDs due to an inefficiency in LD nucleation (Fig.3a).
249 Such cells could also compensate for the lack of tubules and develop alternative
250 mechanisms to facilitate LD assembly [7]. Finally, cells might dynamically shift their ER
251 tubule/sheet ratio pending on metabolic cues, e.g. increase it to generate more LDs (Fig.3a).

252

253 ***Role of seipin in LD nucleation.***

254 Seipin is an ER membrane protein capable of determining the nucleation sites of TG LDs
255 and localizing at the ER-LD junction [31,76–80] (Fig.1c). Seipin possesses two
256 transmembrane domains, with both N- and C-termini facing the cytoplasm and a luminal loop
257 bridging the transmembrane domains [76]. The protein homo-oligomerizes into ring-like
258 structures and the luminal domains of these rings [81,82], of circa 15 nm in diameter [31],
259 resolved by cryo-electron microscopy for human, fly, and yeast, consist of 11, 12, and 10
260 seipin monomers, respectively [36,83,84]. The human and fly monomers possess
261 hydrophobic helices, dipped into the bilayer, and two beta-sheets of four beta-strands each,
262 reminiscent of the sterol-binding protein Niemann-Pick C2 [83,84]. The luminal and
263 transmembrane domains are sufficient to rescue the LD biogenesis defect in seipin-deficient
264 cells, indicating that they are critical for seipin function in LD formation [77].

265

266 The ER membrane curvature and physical chemistry regulate the CNC and the nucleation
267 energy barrier (Figure I-II Box.2). The oligomeric structure of seipin may locally exacerbate
268 topological and biochemical parameters for further decreasing the CNC or the energy barrier
269 [13,29,85,86]. This means that (a) the probability of nucleation will be exponentially much
270 higher at seipins than elsewhere [13] and (b) it becomes thermodynamically more favorable
271 to drive free TGs in membranes into seipin-nucleated LDs than assembling them into new
272 non-seipin LDs (Fig.1) [13]. Seipin is enriched at ER tubules/edges of sheets [11] and likely
273 acts as a membrane curvature sensor or inducer [11] (Fig.3b). Induction of a local curvature
274 by seipin could be a mechanism for inducing the nucleation of TG LDs. Swollen cells can
275 present large onion ring-like ER structures, lacking curvature but bearing seipin [11]. Under
276 these conditions, NLs remain in the ER membrane, and nucleation is severely impeded [11].

277

278 The seipin oligomer structure can also provide a means to locally exacerbate interactions of
279 seipin with lipids or their synthases [9,87–90], constituting subdomains of higher nucleation
280 probability. In particular, seipin can bind to anionic phospholipids such as PA [84] via its
281 beta-sandwich fold, and this might locally regulate both PL and NL synthesis to provide a
282 favorable environment for TG condensation (Fig.2c). Such an environment might prolong the
283 contacts of TG with the oligomer and increase their probability of condensation at seipin

284 (Fig.2c). In simulations, the luminal domain of human seipin traps individual TGs within its
285 ring structure in a model ER bilayer and catalyzes TG clustering at low TG concentrations,
286 where TGs fail to cluster in the absence of seipin [85,86] (Figure I Box.2). At a given TG
287 concentration, TG clusters preferably form inside the seipin ring (Fig. 2c). Serine residues in
288 helices associated with the luminal membrane leaflet and delineating the inner opening of the
289 seipin ring appear critical for TG clustering and subsequent LD formation [86].

290

291 Since curvature is sufficient to trigger the assembly of TG LDs [31,78], the enrichment of
292 seipin at tubules would prevent erratic nucleation events triggered by the sole action of
293 curvature [11]. If a seipin oligomer initiates an LD, it will be more favorable for NLS across a
294 certain distance in the tubule to move to this LD (Fig.2c). Therefore, controlling all LD
295 nucleation events requires that a critical density of seipin oligomers is maintained [31]
296 (Fig.3b,3c). If this condition is not met, regions of seipin-deficient phenotypes will appear
297 within the same cell (Fig.3d). This prediction is demonstrated by experiments where seipin is
298 trapped to the nuclear envelope and LDs forming at seipins have a normal phenotype, while
299 the rest of the ER membrane, with low seipin levels, displays a lipodystrophic phenotype [31]
300 (Fig.3d). These observations indicate that the seipin/tubule ratio needs critical regulation to
301 prevent uncontrolled nucleation (Fig.3c,3d).

302

303 It is more favorable for TGs to move to seipin-nucleated LDs than to assemble into new
304 seipin-negative ones [13]. This is because seipin-LDs have already overcome the nucleation
305 barrier. However, because LDs can be heterogeneous in size, moving TG toward larger LD
306 would be more favorable because of their lower internal **Laplace pressure** [13,91]. To
307 ensure that all nucleated LDs grow, NLS need to be directed towards the smaller LDs,
308 combating the Laplace pressure [31]. Indeed, if seipin is removed from LD-ER junctions,
309 smaller LDs lose TG for larger LDs through the ER bilayer [31]. Finally, given the diversity of
310 NLS, it may be that seipin mainly functions in TG LD biogenesis, but not universally for all
311 NLS [48].

312

313 ***Role of seipin co-workers in LD nucleation.***

314 Recent works indicate that seipin co-operates with and forms an LD assembly complex with
315 promethin/LDAF1 (yeast Ldo 16/45) [78,92–94]. Promethin also forms distinct domains in the
316 ER and several of these foci associate with seipin [78]. Seipin complexes synthesized under
317 lipid-poor conditions do not readily associate with promethin but when NL synthesis is
318 initiated, the fraction of seipins associating with promethin increases [86]. This suggests that
319 NLS facilitate the formation of seipin-promethin complexes. LDs form at sites typically
320 occupied by both seipin and promethin, and upon subsequent LD growth, promethin

321 relocates to the LD surface thanks to its hairpin topology [92], while seipin remains at the ER-
322 LD junction [78].

323

324 Additional players defining the nature of the LD forming ER subdomain, and cooperating with
325 seipin in LD formation, are also involved[95]. In yeast, seipin (Fld1) and nuclear envelope
326 morphology protein 1 (Nem1) localize to the ER, independently of each other, but both mark
327 LD nucleation sites and co-localize to ER-LD contact sites [9]. Nem1 is an activator of the
328 DAG synthesizing Pah1 (mammalian lipin) enzyme. Upon activation of TG synthesis, the
329 association of Fld1 and Nem1 increases, and this recruits the TG synthase Lro1, spatially
330 coupling TG formation and storage sites. Importantly, a catalytically inactive Lro1 does not
331 associate with the Fld1-Nem1 complex, implying that TG is needed for this association. Both
332 Fld1 and Nem1 are required for the subdomains to recruit additional LD biogenesis factors,
333 such as yeast homolog of FIT2 (Yft2), Pex30, and perilipin1 [9].

334

335 **Concluding Remarks**

336 The fundamental physicochemical concepts that underlie the phenomenon of LD nucleation
337 are based on classical principles of thermodynamics and mechanics. When these are
338 combined with experimental findings from *in vitro* model membranes, *in vivo* cell membranes,
339 and *in silico* computational approaches, a comprehensive understanding of this process is
340 starting to emerge. At present, several outstanding questions remain to be addressed, see
341 Outstanding Questions Box. To start with, most LDs are not constituted by a pure, single NL
342 species but by mixtures of them [43]. We understand little how the complex NL chemistry
343 impacts LD nucleation and if LDs of heterogeneous composition are nucleated from a
344 continuous ER network [4,11]. *In vitro*, it is not possible to generate pure SE LDs but in cells,
345 predominantly SE containing NLs can be formed in the presence of excess cholesterol when
346 TG synthesizing enzymes are inhibited. What solubilizes the SE to enable the nucleation of
347 such LDs, is not known.

348

349 Furthermore, is the role of seipin dedicated to TG, and perhaps SE, LD nucleation? What are
350 the specific functions of seipin partners in LD nucleation and how are the LD assembly
351 protein complexes spatiotemporally assembled in different systems? In this context, we also
352 need to understand how ER organization beyond tubules and sheets, such as 3-way
353 junctions, domains involved in protein translocation and folding, etc., impact NL distribution
354 and LD assembly complexes. This, in turn, is related to more physiological questions of LD
355 nucleation mechanisms in different cell types and metabolic conditions: the process in
356 hepatocytes or enterocytes may differ considerably from that in adipocytes or white blood
357 cells. What is foreseeable is that LD nucleation will keep inciting an increasing array of

358 investigators, from biofuels to food production and health care, spurred by megatrends such
359 as pandemic obesity and increasing energy demand.

360

361

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582 **Glossary**

583

584 **Neutral lipids:** hydrophobic apolar molecules, lacking charged groups. Usually optimal
585 forms of energy storage rich in carbon. Can differ between cell types and metabolic states.

586 **Triacylglycerols:** three fatty acids esterified to each of the three alcohol groups of glycerol.
587 Most abundant neutral lipids in eukaryotes that serve for energy and membrane lipid storage.
588 Often referred to as triglycerides. Mostly stored in adipocytes.

589 **Sterol ester:** a fatty acid esterified to the alcohol of a sterol. The second most abundant
590 neutral lipid after triglycerides. Ubiquitous in yeast. In mammals, mostly present in
591 macrophages, adrenals and blood low-density lipoproteins as the storage form of cholesterol.

592 **Surface tension:** energy per area cost for exposing two fluids of different nature. Acts to
593 minimize the interface, which is why droplets are spherical. Oil/water surface tension is
594 decreased by the adsorption of amphiphilic molecules, such as phospholipids or amphipathic
595 proteins, masking the interface. Surface tension of a bilayer is close to zero because of their
596 similar inner and outer aqueous phases. For both monolayers and bilayers, the tighter the
597 phospholipid packing, the lower the surface tension.

598 **Surface pressure:** the lateral pressure acting on a monolayer or a bilayer. Considers the
599 different interactions at the interface, such as those between phospholipids, phospholipids,
600 and neutral lipids, or phospholipids/neutral lipids and water. Lateral pressure is lower when
601 phospholipids are pulled apart or when surface tension is augmented.

602 **Laplace pressure:** the pressure difference across a curved interface. For LDs, proportional
603 to the surface tension of the interface divided by the droplets' curvature. Large droplets have
604 a lower internal pressure than smaller ones, for a given surface tension. Compared with
605 droplets, the Laplace pressure of bilayers is almost zero, because of the low tension.

606 **Ostwald ripening:** leakage of materials due to chemical potential differences. Small
607 particles/droplets of large internal pressure spontaneously lose their molecules for bigger
608 particles/droplets of lower internal pressure, through a continuous medium where the
609 molecules are soluble. Ripening leads to LD heterogeneity and the eventual disappearance
610 of small LDs.

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613

614 **Box1. Basic principles of nucleation phenomena.**

615 *Nucleation is a century-old thermodynamics principle, thoroughly explored theoretically and*
616 *experimentally in diverse contexts [30,96]. Nucleation is the onset of phase separation or*
617 *unmixing of constituents of a blend, e.g. of liquids, gases, or solids. It happens when it*
618 *becomes energetically favorable to decrease the contact between foreign molecules within*
619 *the mixture and it can be triggered by changing a physical chemistry parameter of the*
620 *mixture. Examples of nucleation phenomena are the onset of salt crystallization upon*
621 *reducing water concentration, or the condensation of water molecules from a gas state into*
622 *raindrops, catalyzed by/at dust present in the air. In biology, nucleation emerged during the*
623 *past decade as a crucial step of phase separating phenomena that govern the homeostasis*
624 *of organelles [97]. It is involved in the assembly of phospholipid bilayer-bounded organelles,*
625 *such as the Golgi apparatus [98], but also in the biogenesis of monolayer-encircled*
626 *organelles, such as LDs [13] or membraneless organelles devoid of phospholipids called*
627 *condensates [99]. How and where organelle nucleation is triggered has recently become a*
628 *central question in biology [99].*

629

630 **Box2. The nucleation energy barrier.**

631 *An energy barrier needs to be overcome for nucleation to occur. The lower this energy*
632 *barrier, the higher is the probability to nucleate (Figure I). The height of the energy arises*
633 *from the competition between antagonist contributions involving chemical potentials and*
634 *interfacial energies [13].*

635 **Chemical potentials.** *Molecules constituting a blend tend to demix because mixing*
636 *generally costs energy. For demixing, the interaction of an NL with the bilayer components*
637 *has to cost more energy than the interaction between NLs: the chemical potential of an NL*
638 *within a PL bilayer, $\mu(\text{NL})_{\text{bilayer}}$, needs to be larger than that within LDs, $\mu(\text{NL})_{\text{LD}}$. If this*
639 *condition is unmet, entropy is sufficient to keep NLs mixing with the membrane constituents,*
640 *thereby hampering nucleation. The chemical potential of NLs in membranes $\mu(\text{NL})_{\text{bilayer}}$ is*
641 *likely altered as soon as features of the membrane are varied, e.g. the membrane*
642 *biophysical and physicochemical factors, protein density, and composition, etc. (Figure II).*
643 *Increasing $\mu(\text{NL})_{\text{bilayer}}$ makes the NL-membrane mixing more unfavorable, which decreases*
644 *the energy barrier and promotes nucleation (Fig. 2a). The gap in chemical potentials*
645 *modulates the height of the energy barrier.*

646 **LD interfacial energies.** *Membrane mechanics has several inputs into the energy barrier*
647 *that can work for or against nucleation. There is for instance an energy penalty coming from*
648 *the bilayer leaflet deformation during nucleation; for nucleation to occur, the bilayer leaflets*
649 *have to be unzipped and its monolayers deformed; this can be combated by the rigidity of the*
650 *leaflets. Reducing rigidity would reduce the cost of deformation energy and facilitate*

651 *nucleation. Rigidity is often increased by PL saturation, cholesterol, and possibly sterol*
652 *esters, which would disfavor nucleation. Another membrane input is surface tension;*
653 *increasing the bilayer surface tension, by pulling or stretching on the bilayer, would disfavor*
654 *bilayer deformation and nucleation [40,41]. In vitro, LD nucleation requires low bilayer*
655 *tension.*

656

657

658

659 **Figure legends**

660

661 **Figure 1:** a) Schematic illustration of the esterification of diacylglycerol or cholesterol by
662 diacylglycerol O-acyltransferase 1 (DGAT1), or acyl-coenzyme A: cholesterol acyltransferase
663 1/sterol O-acyltransferase 1 (ACAT1/SOAT1), or membrane-bound O-acyl transferase
664 (MBOAT). The synthesized neutral lipid exits the enzyme through a lateral gate facing the
665 bilayer hydrophobic core. b) Molecular structures of triolein and sterol oleate, the major
666 triglycerides and sterol esters in neutral lipid stores. c) Examples of other neutral lipids with
667 ester functions. d) Examples of other neutral lipids with non-ester functions.

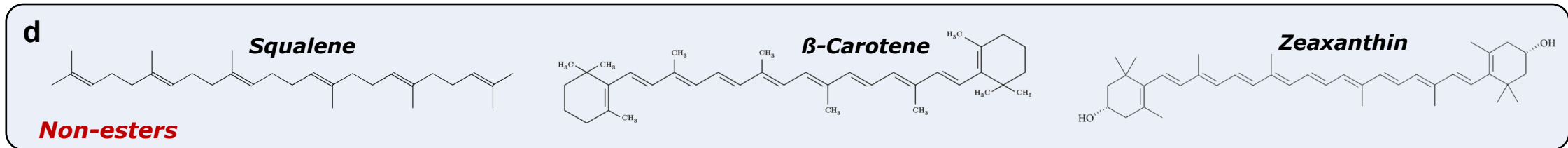
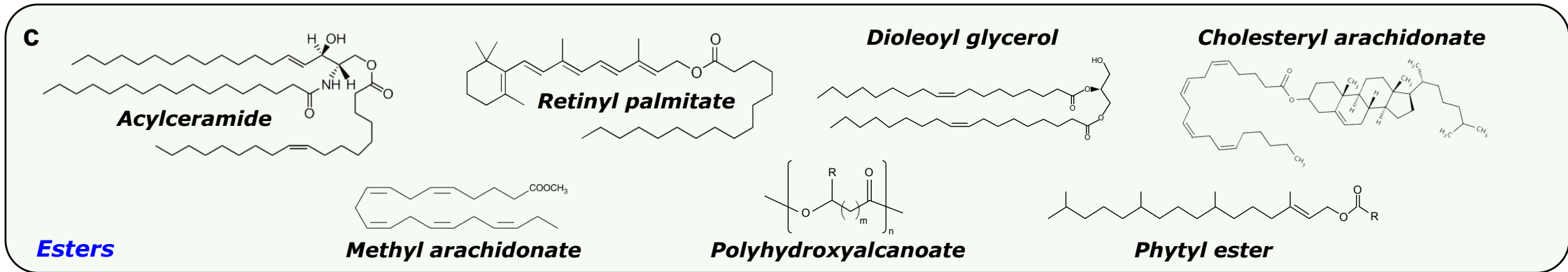
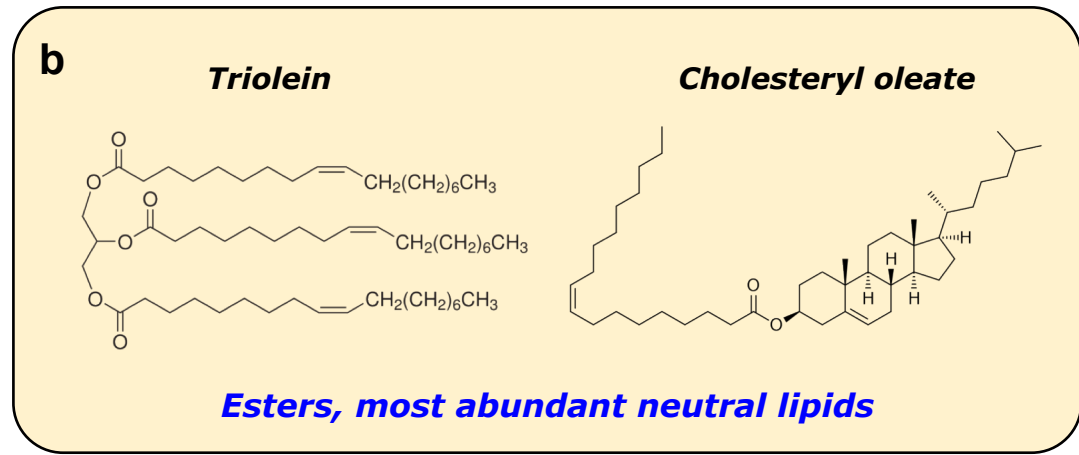
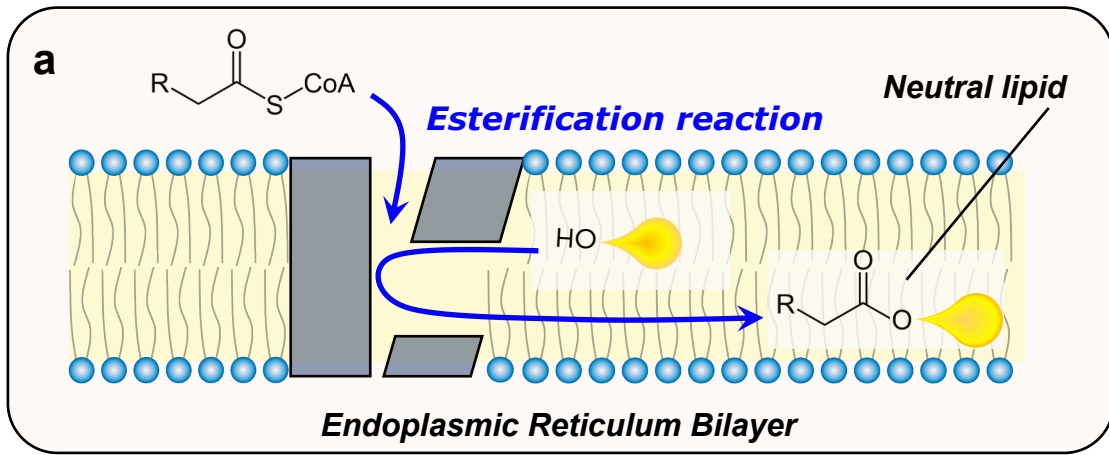
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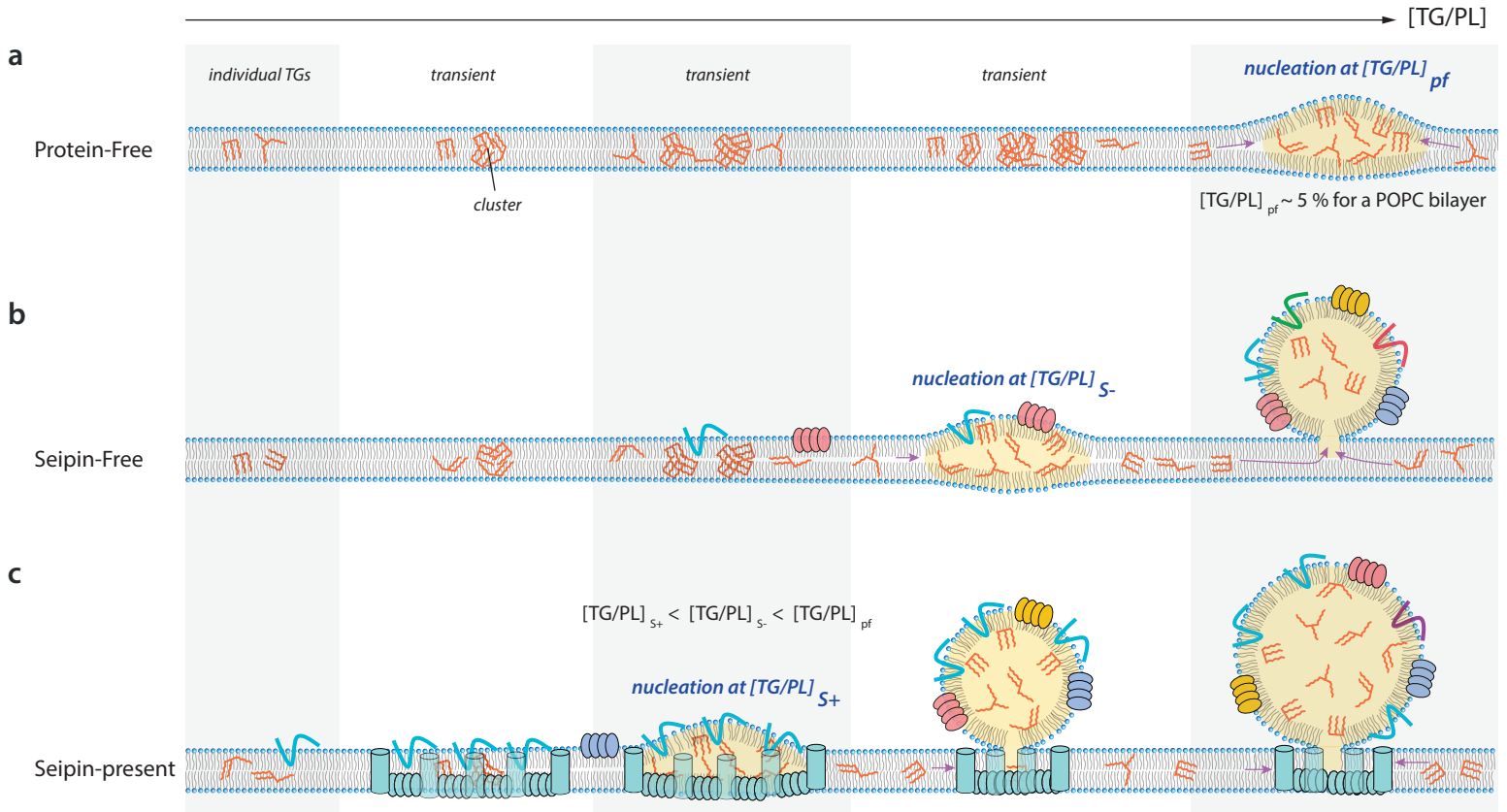
669 **Figure 2.** Schematic illustration of the condensation of triglycerides (TG) in protein-free (pf),
670 seipin deficient (S-), and in seipin containing conditions (S+). Increasing the concentration of
671 TG per phospholipids, [TG/PL] leads to the formation of transient clusters that are bigger
672 steadier for larger TG concentrations. Nucleation, i.e. TG condensation, happens at a critical
673 concentration around ~5% in protein-free conditions. Proteins, in particular seipin, might
674 decrease the critical nucleation concentration. ER: endoplasmic reticulum, LD: lipid droplet,
675 AH: amphipathic helix, Plin1: perilipin 1, LDAF1: lipid droplet factor 1.

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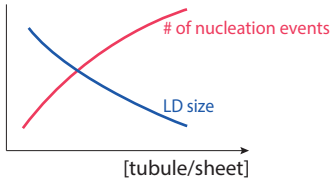
677 **Figure 3.** a) Illustration of the evolution of TG LD nucleation frequency and LD size pending
678 on ER tubule/sheet ratio. b) Illustration of the enrichment of seipin in tubules vs. sheets. In
679 seipin deficiency, more TG LD nucleation events happen at tubules. c) Evolution of
680 nucleation frequency pending on the seipin/tubule ratio. At low seipin dose, a lipodystrophic
681 phenotype is observed. d) In a single cell, seipin is trapped at the nuclear envelope
682 membrane where a normal LD biogenesis phenotype is observed. In the rest of the cell
683 ("periphery"), a seipin deficient and lipodystrophic phenotype is observed. A critical density of
684 seipin is required throughout the cell to prevent the undesired lipodystrophic phenotype.
685 Figure adapted from Figure 7 of Salo et al. *Developmental Cell* 2019 [31].

686

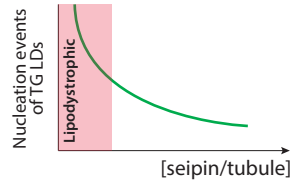




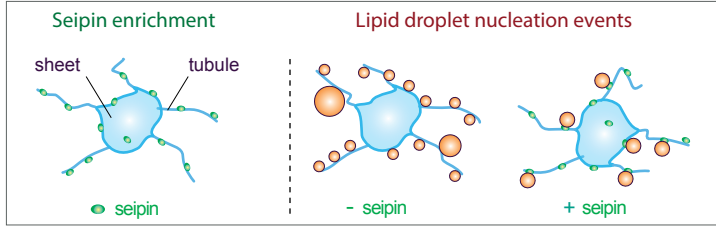
a Triglyceride(TG) concentration constant



c



b



d

