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# Mechanisms of protein targeting to lipid droplets: A unified cell biological and biophysical perspective

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## ► To cite this version:

Aymeric Chorlay, Ravi Dhiman, Stefanie Caesar, Abdou Rachid Thiam, Bianca Schrul. Mechanisms of protein targeting to lipid droplets: A unified cell biological and biophysical perspective. *Seminars in Cell and Developmental Biology*, 2020, 219 (4), 10.1016/j.semcdb.2020.03.004 . hal-03046304

**HAL Id: hal-03046304**

**<https://hal.science/hal-03046304v1>**

Submitted on 15 Dec 2022

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1 **Mechanisms of protein targeting to lipid droplets: A unified cell biological and**  
2 **biophysical perspective**

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24 **Abstract**

25 Lipid droplets (LDs), or oil bodies in plants, are specialized organelles that primarily serve as  
26 hubs of cellular metabolic energy storage and consumption. These ubiquitous cytoplasmic  
27 organelles are derived from the endoplasmic reticulum (ER) and consist of a hydrophobic  
28 neutral lipid core - mainly consisting of triglycerides and sterol esters - that is encircled by a  
29 phospholipid monolayer. The dynamic metabolic functions of the LDs are mainly executed  
30 and regulated by proteins on the monolayer surface. However, its unique architecture puts  
31 some structural constraints on the types of proteins that can associate with LDs. The lipid  
32 monolayer is decorated with either peripheral proteins or with integral membrane proteins  
33 that adopt a monotopic topology. Due to its oil-water interface, which is energetically costly,  
34 the LD surface happens to be favorable to the recruitment of many proteins involved in  
35 metabolic but also non-metabolic functions. We only started very recently to understand  
36 biophysical and biochemical principles controlling protein targeting to LDs. This review aims  
37 to summarize the most recent findings regarding this topic and proposes directions that will  
38 potentially lead to a better understanding of LD surface characteristics, as compared to  
39 bilayer membranes, and how that impacts protein-LD interactions.

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47 **Keywords:** protein-lipid interaction, amphipathic helix, monotopic hairpin topology,  
48 phospholipid monolayer, PEX19, endoplasmic reticulum,

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54 **1. Introduction: The unique architecture of lipid droplets and implications for**  
55 **associated proteins**

56 Cells and their organelles are surrounded by phospholipid membranes, which serve as  
57 protective barriers and segregate diverse sets of proteins into compartments with distinct  
58 physicochemical properties. Over the last decades, there has been a tremendous increase in  
59 our knowledge about how membrane proteins are targeted to and integrated into  
60 phospholipid bilayer membranes. Membrane proteins can associate with the membrane  
61 either in an integral or peripheral fashion. Peripheral proteins are not stably embedded into  
62 the membrane and interact with the membrane *via* other proteins or phospholipids, often in a  
63 reversible manner, while integral proteins are stably embedded into the phospholipid bilayer  
64 and can adopt different types of topologies. Bitopic proteins traverse the membrane once  
65 with a hydrophobic transmembrane domain and expose soluble domains on both sides of the  
66 membrane. Polytopic membrane proteins span the membrane multiple times. Monotopic  
67 proteins do not fully traverse the membrane [1]. They stably integrate into the membrane *via*  
68 either hydrophobic hairpin (HP) domains or amphipathic helices (AH) and expose all soluble  
69 domains towards one side of the membrane.

70 Lipid droplets (LDs) are unique organelles in the sense that they are the only  
71 organelle decorated by a phospholipid monolayer, covering the LD neutral lipid oil core. This  
72 interfacial architecture raises new biophysical questions in terms of protein-lipid interactions,  
73 which are so far not well documented. Several proteomic studies revealed a set of about  
74 100-150 LD proteins in mammalian cells and about 40 LD proteins in yeast cells [recently  
75 reviewed in 2]. The protein composition varies depending on the cell type, the metabolic  
76 status and also on the method used for the isolation of LDs. Proteins that have been  
77 annotated as endoplasmic reticulum (ER)- and mitochondria-resident proteins are frequently  
78 detected in LD proteomes. While such proteins may be considered contaminations from the  
79 biochemical LD isolation from cells, probably due to the multi-contacting organelle  
80 particularity of LDs, many of them indeed show a dual subcellular localization. Therefore it is  
81 important to validate the localization of the candidate proteins to LDs by independent  
82 methods, such as fluorescence microscopy. For many LD proteins a metabolic function on  
83 LDs could be verified. It will, however, be a major goal for the next decades to  
84 comprehensively understand how the LD proteome regulates LD functions. Current research  
85 focuses on understanding this organelle on a broader (patho-) physiological and molecular  
86 level. Many fundamental questions about LD biogenesis and functions are still unanswered.  
87 Questions at the center of this review are: How are proteins targeted to LDs? What features  
88 make the LD phospholipid monolayer surface distinct from the external monolayer of a  
89 bilayer membrane, especially that of the ER? How do proteins sense these differences?

90

## 91 **2. Lipid droplet biogenesis from the ER membrane**

92 LD biogenesis is triggered by several metabolic or stress conditions and its main steps have  
93 been described in several recent reviews [2-6] (Figure 1). Biogenesis of LDs is initiated by a  
94 cascade of biochemical reactions taking place at the ER membrane and transforming fatty  
95 acids and/or cholesterol into final neutral lipid products, triglycerides (TG) and sterol esters  
96 (SE), respectively. Due to their hydrophobicity, these molecules are hidden in the interstice of  
97 the two phospholipid monolayer leaflets composing the ER membrane. When a critical  
98 concentration of neutral lipids is reached within the bilayer, the molecules segregate from the  
99 bilayer by phase separation [7], i.e. condensation and separation of the neutral lipids from  
100 the phospholipid acyl chains of the bilayer. This mechanism leads to the nucleation of a  
101 neutral lipid lens within the bilayer [8-10]. The nucleated lens grows by adsorbing more  
102 neutral lipids until becoming a spherical LD that buds off and subsequently detaches from the  
103 ER taking with it phospholipids from the cytosolic leaflet. The LD formation process proceeds  
104 *via* four steps: nucleation, growth, budding, and detachment [7], detailed below.

105 The mechanisms of nucleation are currently unknown but may take place at specific  
106 ER regions [11, 12]. Proteins that have been located to early LD biogenesis sites include  
107 seipin and its interacting partner Promethin (or LDAF1) [12-15], Acyl-CoA synthetase 3  
108 (ACSL3) [16], and PEX30 in yeast or the multiple C2 domain containing transmembrane  
109 protein (MCTP2) in mammals [11, 12]. These proteins can define LD nucleation sites by  
110 providing local membrane environment (e.g. lipids, curvature) favorable for the neutral lipid  
111 condensation [14, 15]. How exactly the nucleation process occurs remains to be elucidated.  
112 Modulation of the local membrane composition or biophysical features such as bending,  
113 surface tension, or curvature at these regions may facilitate LD nucleation [9, 17-20].  
114 Curvature is induced by many ER proteins. Reticulons for example are integrated into  
115 membranes in an asymmetric fashion, occupying more space in the cytosolic leaflet of the  
116 bilayer than in the luminal leaflet and thereby inducing membrane curvature and ER  
117 tubulation. Indeed, the structural integrity of the ER membrane appears to be crucial for  
118 correct LD biogenesis as depletion of atlastin or REEP1 results in aberrant LD sizes [21-23].  
119 PEX30 and MCTP2, which localize to LD biogenesis sites, show structural similarity to  
120 reticulons [11].

121 The growth step is strongly regulated by the ER-resident transmembrane protein  
122 seipin [13, 14, 24-28]. Tiny nucleated LDs are under higher internal pressure than pre-  
123 existing ones; they will consequently tend to dissolve their content back to the bilayer. Seipin  
124 counterbalances this back flow by possibly having an active function in incorporating neutral  
125 lipids into the nascent LDs [14, 27, 28].

126 The budding step is strongly dependent on ER membrane tension and phospholipid  
127 composition [9, 18, 19]. These parameters alter the packaging of neutral lipids into LDs and

128 thereupon control LD size [9, 29]. The emergence side of LDs to the cytosol is regulated by  
129 an asymmetry in the ER membrane composition as well as curvature [17-19]. Keeping a  
130 continuous emergence of LDs to the cytosol is ensured by refilling the cytosolic ER leaflet  
131 with phospholipids under lipogenesis conditions [17, 18]. This keeps the cytosolic monolayer  
132 always ready to cover emerging LDs with phospholipids and to balance its phospholipid  
133 amount with that of the ER luminal leaflet. Such a process is necessary to maintain ER  
134 homeostasis. An alternative means to keep the ER monolayer leaflets balanced is to deplete  
135 phospholipids from the luminal monolayer while a LD is emerging to the cytosol. Such  
136 mechanism could be mediated by the fat storage-inducing transmembrane protein 2 (FIT2),  
137 which is suspected to act as a lipid phosphatase at the luminal membrane surface [30, and  
138 preprint manuscript: doi: <https://doi.org/10.1101/291765>] and whose depletion results in  
139 defects of LD budding from the ER [10, 31]. In the luminal leaflet, FIT2 could convert  
140 phosphatidic acid to diacylglycerol, which leaves the monolayer and solubilizes into the  
141 forming LD TG core. This mechanism would compensate the decrease in phospholipid  
142 number on the cytosolic monolayer, induced by LD emergence.

143 Finally, the mechanisms of the detachment process are still not known. An indirect  
144 proof of the occurrence of detachment is the observation by live cell imaging of the  
145 reconnection of a LD subpopulation to the ER membrane, mediated by complex protein I  
146 (COPI) [32, 33]. This reconnection is based on the spatial distribution of protein reporters  
147 such as GPAT4, which diffuses from the ER to LD in a COPI-dependent manner [33].

148

### 149 **3. Establishing the lipid droplet proteome: class I and class II proteins**

150 Very little is known about the underlying principles involved in protein targeting to LDs. This  
151 tremendous gap in knowledge mainly stems from the previous lack of a comprehensive  
152 determination of the LD proteome and the lack of obvious organelle-targeting sequences.  
153 Nonetheless, previous studies have pinpointed two classes of proteins that physically  
154 localize to LDs (Figure 1): Class I proteins are initially inserted into the ER membrane from  
155 where they can partition to LDs, potentially *via* lateral diffusion. They often bear so-called  
156 hydrophobic hairpin (HP) domains, which stably integrate into the bilayer membrane in a  
157 monotopic topology that presumably enables the membrane partitioning. Class II proteins  
158 generally target LDs from the cytosol. They mostly have unfolded amphipathic helices (AH)  
159 that fold to the LD surface when it is accessible. Alternatively, class II proteins can insert into  
160 the phospholipid monolayer through lipid-anchors. So far, these are the main classes of  
161 proteins found on LDs. However, in light of the numerous contact sites established between  
162 LDs and other organelles, such as peroxisomes and mitochondria [34, 35], proteins may  
163 target to LDs from these organelle counterparts, and therefore additional classes may exist  
164 [36]. For example, in *Arabidopsis thaliana*, the TAG lipase SDP1 leaves peroxisomes and

165 partitions to the oil bodies during post-germinative growth [37]. Similarly, fatty acyl-CoA  
166 reductase 1 (FAR1) differentially localizes to peroxisomes and LDs under certain metabolic  
167 conditions [38]. Whether such proteins partition from peroxisomes to LDs directly or *via* the  
168 ER remains to be investigated. Finally, proteins might be directly translated on the LD  
169 surface [recently discussed in 39].

170

## 171 **4. Protein targeting to lipid droplets**

### 172 **4.1 Conventional protein targeting pathways**

173 Subcellular organelles are characterized by their unique subset of proteins that are either  
174 imported into the lumen of these organelles or integrated into their limiting membranes. A  
175 major task of the cell is to achieve specificity in protein targeting to the correct destination  
176 organelle. Research over the last decades revealed some underlying principles in protein  
177 targeting that are shared by several organelles and conserved across species [40-42]: Signal  
178 sequences within the newly synthesized proteins contain information that is specifically  
179 recognized by soluble, cytosolic factors leading to the formation of pre-insertion complexes  
180 that in turn recognize membrane-receptors on the surface of the destination organelle. For  
181 translocating proteins across membranes or for the insertion of hydrophobic integral, bitopic  
182 or polytopic, proteins into the membrane, protein-conducting channels such as the Sec61  
183 translocon in the ER membrane or the TIM/TOM complexes in the mitochondrial membranes  
184 are usually required. They form pores within the phospholipid bilayer membranes enabling  
185 the passage of soluble domains through the hydrophobic membrane and can assist in the  
186 lateral integration of hydrophobic membrane-spanning segments.

187 Protein targeting can occur during translation on cytosolic ribosomes (co-translational  
188 targeting) or after translation has been completed (post-translational targeting). Co-  
189 translational protein targeting to the ER has been studied for decades and to atomic  
190 resolution [43]. Most secretory and ER-resident proteins contain N-terminal signal sequences  
191 that consist of an N-terminal n-region, which is usually enriched in positively charged amino  
192 acids, followed by a hydrophobic h-region and a c-region, which may contain consensus  
193 sequences allowing the cleavage of the signal sequence in the ER lumen by signal  
194 peptidase. Such signal sequences are usually recognized by the signal recognition particle  
195 (SRP) as soon as they emerge from the translating ribosome in the cytosol (Figure 2A). The  
196 SRP-ribosome-nascent chain complex is then co-translationally recruited to the ER  
197 membrane where it interacts with the SRP-receptor and the translocation channel.  
198 Translation of the protein is continued to allow direct translocation across or insertion of the  
199 nascent protein into the ER membrane. Coupling of protein synthesis and membrane  
200 insertion is an elegant way to continuously shield hydrophobic domains of the nascent  
201 protein from the aqueous cytosol and to protect the protein from uncontrolled aggregation.

202 For LD-destined membrane proteins no signal sequences or components that could  
203 mediate the direct insertion of newly synthesized proteins into the limiting monolayer  
204 membrane of LDs have been discovered. Instead, many stably membrane-integrated LD  
205 proteins are initially inserted into the ER membrane before they partition to the LD surface  
206 (class I proteins): Examples include AUP1 [44, 45], Caveolin-1 [46], DGAT2 [47], AAM-B and  
207 UBXD8 [48]. These monotopic ER/LD proteins do not contain cleavable N-terminal signal  
208 sequences, which raises the question whether they employ any of the conventional ER-  
209 targeting pathways.

210 For oleosins, major proteins of plant LDs, it has been shown that they integrate into  
211 the ER membrane in a SRP- and Sec61 translocon-dependent manner [49, 50]. The  
212 hydrophobic hairpin region of these proteins, however, is much longer than those of other  
213 short hairpin proteins, which seem to integrate into the ER membrane without the assistance  
214 of a translocation channel. For most class I proteins it is unknown how they are recognized,  
215 which factors mediate the specific insertion of the protein into the ER membrane, and how  
216 these processes are regulated.

217 Alternative targeting routes to the ER include the transmembrane recognition  
218 complex (TRC) system, which mediates the post-translational membrane insertion of newly  
219 synthesized tail-anchored (TA) membrane proteins [51]. In mammals, soluble TRC40 binds  
220 the hydrophobic C-terminal domain, which only emerges from the ribosome once translation  
221 has been completed, and together with membrane resident receptors mediates the  
222 integration of this domain into the membrane. Whether the assistance of a translocon is  
223 required for the membrane insertion of all TA-proteins is an open question in the field. Very  
224 recently, a third targeting route for ER-destined proteins, the SRP-independent (SND)-  
225 pathway, has been discovered [52, 53]. The SND-pathway likely acts in parallel and as back  
226 up to the SRP- and the TRC-systems. Interestingly, the position of the hydrophobic domain  
227 within the newly synthesized protein seems to be a crucial feature determining with which  
228 targeting system it preferentially engages and the SND system apparently prefers cargo  
229 proteins containing rather central hydrophobic domains [52]. It will be interesting to test  
230 whether short hairpin proteins with central hydrophobic domains employ the SND pathway  
231 for their initial insertion into the ER membrane and if so, which features of their hydrophobic  
232 domains are critical for their recognition.

233

#### 234 **4.2 PEX19-mediated targeting of LD-destined class I proteins to the ER**

235 A recent study describes the ER targeting pathway for newly synthesized UBXD8 [54]. The  
236 post-translational targeting of UBXD8 to the ER is independent of the canonical ER targeting  
237 pathways and its insertion into bilayer membranes does not depend on a protein-conducting  
238 channel such as the Sec61 complex. This, however, is not surprising, as no soluble domains

239 of the protein need to be translocated across the membrane to establish a monotopic hairpin  
240 topology. Instead, specific targeting to the correct destination organelle is established by a  
241 physical interaction of newly synthesized UBXD8 with the soluble protein PEX19. Together  
242 with the membrane-embedded protein PEX3, PEX19 is essential for the correct insertion of  
243 UBXD8 into distinct subdomains of the ER membrane [54] (Figure 2A). Interestingly, PEX19  
244 and PEX3 are known as essential peroxisome biogenesis factors and they also mediate the  
245 post-translational insertion of newly synthesized peroxisomal membrane proteins (PMPs)  
246 into peroxisomal membranes [55]. Together, these observations raise the question why two  
247 distinct organelles would share protein targeting machinery as this poses a potential risk for  
248 protein mis-sorting.

249         LDs and peroxisomes fulfill complementary functions in lipid metabolism [56]: LDs  
250 store neutral lipids that are hydrolyzed into fatty acids under catabolic conditions. Fatty acids  
251 can then in turn be oxidized in peroxisomes. Conversely, peroxisomes synthesize ether  
252 lipids, which can be esterified and stored in LDs under anabolic conditions. Both organelles  
253 can originate from the ER membrane [57] and physically interact with each other [35, 58, 59].  
254 Sharing PEX19 as a common targeting factor may facilitate a coordinated (protein-)  
255 biogenesis of both organelles, which may be relevant for the timely adaptation of their  
256 function to changing metabolic conditions such as nutrient availability [56]. Certainly, it will be  
257 important to identify the full PEX19 cargo spectrum on LDs and the respective functions of  
258 these proteins with regard to lipid metabolism to verify this hypothesis.

259         In addition to ER/LD-resident UBXD8, also some RHD-containing proteins such as  
260 Arl6IP1 can employ the PEX19/PEX3 machinery for their post-translational insertion into the  
261 ER [60]. These proteins, however, do not partition from the ER to the LD membrane  
262 indicating that PEX19 function is not restricted to LD- or peroxisome-destined membrane  
263 proteins. The authors furthermore suggest that shaping of the ER membrane by RHD-  
264 proteins and peroxisome biogenesis may be coordinated [60]. This is an interesting  
265 hypothesis since evidence suggests that the biogenesis of peroxisomes and LDs may be  
266 spatio-temporarily coordinated at specific ER subdomains [57]. Interestingly, UBXD8 is first  
267 inserted into distinct subdomains of the ER in a PEX19/PEX3-dependent fashion [54] but  
268 whether these are LD biogenesis sites is unknown. In *Saccharomyces cerevisiae*, deletion of  
269 *pex3* results in smaller and fewer lipid droplets and, in addition, alters the composition of LDs  
270 compared to wild type cells [61]. *ubx2Δ* yeast cells also show changes in neutral lipid  
271 metabolism such as reduced TAG levels and thus resulting in smaller LDs. The authors  
272 attributed this reduction in TAG levels and aberrant LD biogenesis as being a consequence  
273 of the mislocalization of phospholipid: diacylglycerol acyltransferase (Lro1) [62].  
274 Complementation of *ubx2Δ* cells with the mammalian homologue UBXD8 restored the TAG

275 levels pointing towards a conserved function of UBXD8/UBX2 in yeast and mammals [62]. In  
276 mammalian cells, however, UBXD8 regulates LD turnover by reducing ATGL activity, the  
277 rate-limiting enzyme in lipolysis [63]. Whether LD-destined PEX19 cargo proteins such as  
278 UBXD8 may play an active role in initiating and/or stabilizing ER domains where organelle  
279 biogenesis is initiated remains to be investigated. It would be interesting to test whether  
280 knock-out or mislocalization of UBXD8 in mammalian cells affects the localization of any  
281 early LD biogenesis factors such as acyltransferases.

282 Finally, the question arises how such machinery, which is responsible for targeting  
283 proteins to multiple different destinations including ER/LDs and peroxisomes, deals with  
284 potential protein missorting. First mechanistic insight stems from the observation that  
285 farnesylation of PEX19 is essential to facilitate correct ER and LD localization of UBXD8 [54],  
286 while it is dispensable for peroxisome biogenesis from the ER [64]. In the absence of wild-  
287 type PEX19 in cells, UBXD8 mainly mislocalizes to mitochondria instead of residing in the  
288 ER from where it can partition to LDs, and the expression of a non-farnesylated version of  
289 PEX19 does not rescue this phenotype [54]. Interestingly, strong overexpression of non-  
290 farnesylated PEX19 in a wild-type background results in a dominant-negative effect, such  
291 that a fraction of UBXD8 is recruited to peroxisomes [54]. A model in which distinct cargo  
292 types (peroxisome *versus* ER/LD-destined) differentially bind to PEX19 and control the  
293 exposure of the farnesyl moiety to ensure organelle-specific protein insertion alongside with  
294 an alternative model for PEX19-mediated protein sorting within the ER membrane has  
295 recently been discussed in detail [56]. A systematic comparison of the interaction sites of  
296 ER/LD- and peroxisome-destined proteins with PEX19 would allow the delineation of  
297 conserved features that are important for selective recognition and specific organelle  
298 targeting by PEX19. Likewise, simultaneous tracking of ER/LD- and peroxisome-destined  
299 proteins during their early biogenesis steps would reveal whether they become segregated  
300 prior to their insertion into distinct membranes or after they have been inserted into the same  
301 domains of the ER membrane.

302

#### 303 **4.3 ER-to-LD partitioning: Structural aspects of class I proteins**

304 Sorting LD-destined membrane proteins from the ER bilayer membrane to the LD surface is  
305 a logistic challenge for the cell. Partitioning is likely to occur via contiguous ER and LD  
306 membranes potentially during the emergence of LDs from the outer ER leaflet or alternatively  
307 at later stages when LDs form physical contact sites with the ER. Biophysical properties of  
308 the ER bilayer and the LD monolayer membrane likely create a first selection barrier  
309 controlling which type of proteins are able to partition between these membranes [65, 66].  
310 LDs cannot accommodate proteins with bilayer-spanning transmembrane regions as the LD  
311 monolayer membrane basically reflects only half of a bilayer membrane. Proteins with

312 hydrophilic luminal domains can also not partition to the LD surface, as the exposure of such  
313 domains into the hydrophobic neutral lipid core is energetically unfavorable [65]. Thus, a  
314 monotopic protein topology is probably the most basic criterion to allow ER-to-LD protein  
315 partitioning (Figure 2B). However, not all monotopic proteins partition to LDs. Members of the  
316 reticulon and REEP families also traverse the outer leaflet of the ER bilayer twice to adopt a  
317 hairpin-like or wedge-shaped topology with their N- and C-termini oriented towards the  
318 cytosol, yet, they do not partition to LDs and solely localize to the ER [67, 68].

319

320 We are only at the beginning to understand, which intrinsic features of class I proteins  
321 enable bilayer-to-monolayer partitioning. Structural information about monotopic proteins and  
322 their membrane-embedded domains is still limiting [1] and even the exact position of the  
323 membrane-embedded domains within the proteins has been determined experimentally for  
324 only a few LD-localized proteins such as DHRS3 [69]. In contrast, sequence elements that  
325 are essential for LD-localization have been revealed for a number of proteins and they often  
326 overlap with elements that are also required for the initial insertion of class I proteins into the  
327 ER membrane, suggesting that once the protein is correctly inserted to the ER it has intrinsic  
328 capacity to also partition to the LD surface. Often there is a helix-breaking proline in the  
329 middle of the hydrophobic region, which may cause a kink or turn in the conformation thereby  
330 favoring a monotopic topology. Mutation of this residue changes the subcellular localization  
331 of some class I proteins. For example, AUP1 with a mutated proline-valine-glycine sequence  
332 stayed in the ER instead of being targeted to LDs. Since this mutation also caused a  
333 conformational change from a hairpin topology to a transmembrane protein, basic topology  
334 may restrict the partitioning. However, arginines flanking the hydrophobic domain are  
335 important for AUP1 partitioning but not for preserving its monotopic topology [44]. A central  
336 helix-breaking residue is not always essential for LD targeting. Some class I proteins with an  
337 N-terminal hydrophobic sequence, such as AAM-B, did not change their localization as a  
338 consequence of mutating the central proline residue [70], suggesting that additional features  
339 are important. Regions with positively charged residues or amphipathic helices adjacent to  
340 the hydrophobic hairpin region may influence the topology and the correct LD targeting of  
341 class I proteins.

342 Furthermore, the length of the hydrophobic hairpin regions may be critical for  
343 determining ER-to-LD partitioning. While class I proteins such as AMM-B, UBXD8 and AUP1  
344 contain short hydrophobic hairpin domains of about 20-30 amino acids [44, 70], plant  
345 oleosins have a large central hydrophobic hairpin domain of about 72 amino acids with a  
346 conserved proline knot motif - a triad of prolines - that is essential for partitioning of the  
347 protein from the ER to the oil body [50, 71, 72]. X-ray footprinting together with mass  
348 spectrometry revealed a solvent accessibility map of S3 oleosin from *A. thaliana* and

349 provided experimental evidence that the proline knot and half of the hydrophobic region is  
350 located far from the phospholipid monolayer, which delineates the aqueous phase and  
351 neutral lipid core [73]. These hairpin domains are longer than the thickness of a bilayer and  
352 their accommodation in bilayers will thus cause membrane stress due to hydrophobic  
353 mismatch [74] (see also section 4.5). Since the LD thickness is infinite, relocalization of the  
354 proteins to the LD surface can be a more favorable state and a way to release bilayer stress.

355 Not all class I proteins target to LDs with a hydrophobic hairpin domain. Pataki *et al.*  
356 recently identified a range of monolayer integrated proteins (MIPs) on LDs that biochemically  
357 show similarities with stably integrated transmembrane proteins as they are resistant to  
358 extraction using high salt or alkaline carbonate. Interestingly, probing the solvent accessibility  
359 of each individual amino acid within the hydrophobic region of the class I protein DHRS3 as  
360 well as molecular dynamics simulations revealed an amphipathic interfacial alpha-helical  
361 membrane anchor, a motif that is potentially shared also by other class I MIPs [69]. Class I  
362 proteins could also use a combination of AHs with hydrophobic domains or basic  
363 hydrophobic helices or stretches. This is probably the case for PLIN1, which has a four-helix  
364 bundle on its C-terminus that likely unzips to associate with the ER bilayer and that alone is  
365 sufficient to localize to the ER [75, 76]. The concomitant association of these four AHs, in  
366 addition to the 11mer domain, proffers to PLIN1 a stable membrane association.  
367 Consequently, and in contrast to PLIN2, PLIN3 or PLIN4, PLIN1 is solely found to exclusively  
368 localize to the ER and LDs [75] and therefore is probably a class I LD protein. Other proteins  
369 might follow PLIN1 and DHRS3 in this list of non-HP-containing class I proteins.

370  
371 Once class I LD proteins are inserted into the ER membrane, not all of them partition to LDs  
372 with the same efficiency. Moreover, some of these proteins serve important functions within  
373 the ER membrane as well, suggesting that ER-to-LD partitioning must be regulated (Figure  
374 2B). Despite the intrinsic capability of a protein to reside in two different types of membranes  
375 with distinct physico-chemical environments, regulatory mechanism could depend on i) active  
376 recruitment of LD-destined protein populations, or ii) ER-resident proteins, which could  
377 sequester certain proteins capable of partitioning to the LD surface from the ER - UBAC2, for  
378 example, physically interacts with UBXD8 in the ER membrane thereby restricting the  
379 population of UBXD8 that is free to partition to LDs [63] - or iii) dual topologies (for example  
380 bitopic vs. monotopic), enabling only adequate subpopulations of a protein to traverse to the  
381 LD monolayer [38].

382 Furthermore, it has been shown that not all LDs in a cell are necessarily identical in  
383 their protein and lipid composition [4, 77-79]. The existence of LD subpopulations may reflect  
384 differences in metabolic stages and basic biological functions between LDs. TAG-  
385 synthesizing and certain phospholipid-modifying enzymes may be required during early steps

386 in the biogenesis process and under anabolic conditions, while lipases such as ATGL should  
387 be recruited and activated on the LD surface under LD turn-over and catabolic conditions.  
388 Likewise, FAR1 is a dual-topology protein found on LDs and peroxisomes depending on  
389 metabolic conditions [38], and the *Arabidopsis* TAG lipase SDP1 migrates from peroxisomes  
390 to oil bodies during post-germinative growth [37]. The *Drosophila* protein CG2254/Ldsdh1  
391 partitions from the ER to only a subset of LDs [80]. How cells manage to generate LDs with  
392 different surface and volume chemistry as well as protein content within a single cell remains  
393 to be elucidated. It is conceivable that the initial insertion of class I proteins into the ER  
394 membrane and selective ER-to-LD partitioning are inter-dependent. For newly synthesized  
395 UBXD8 it has been shown that it is inserted into distinct subdomains of the ER membrane  
396 [54] and such spatially restricted membrane integration could support the sorting and  
397 segregation of class I proteins to LDs.

398

#### 399 **4.4 Direct protein targeting to LDs from the cytosol: Structural features of class II** 400 **proteins**

401 Class II proteins target LDs directly from the cytosol. Until now, it is not known whether LD  
402 localization can be facilitated by a receptor-mediated process but most of these proteins bear  
403 AH binding motifs or lipid anchors, which can directly interact with LD monolayer  
404 membranes. AHs of class II LD proteins are usually unfolded in solution and fold into helical  
405 structures upon contact with the cytosol-membrane interface. For example, PLIN2-4  
406 preferentially bind to monolayer membranes *via* their 11mer repeat AH [76, 81, 82].

407 The binding of AHs to LDs is influenced by several factors on both, the protein and  
408 the membrane sides. On the protein side, the amino acid sequence is critical but predictions  
409 for “genuine” LD AH sequences are difficult, if ever possible. A few features, which modulate  
410 protein binding to LDs in general, have been identified and they are based on the AH  
411 hydrophobic degree, i.e. aggregated hydrophobic residue count, and the hydrophobic  
412 moment which is an index of amphiphilicity, indicating how well the hydrophobic and  
413 hydrophilic amino acids are separated within an alpha helical structure [83]. The same  
414 hydrophobic moment can be obtained by different AH topologies: a tiny hydrophobic face but  
415 dense in bulky hydrophobic residues or an AH with the same length but with a large  
416 hydrophobic face with non-bulky hydrophobic residues (Figure 3A). An AH monomer  
417 repeated many times conserves the hydrophobic moment of the monomer but has higher  
418 hydrophobicity (Figure 3C). These two parameters are varied by AH amino acid sequence,  
419 length, and lateral protein-protein interactions. A good example for these considerations is  
420 reflected by the binding mode of PLIN4 (AH of ~1200aa). It is devoid of bulky residues but its  
421 exceptional length (29 tandem 33-mer repeats with a length of 140 nm) determines its stable  
422 LD association, which is further stabilized by possible lateral AH-AH interactions that are

423 mediated by charges on the hydrophilic faces of the AHs [84] (Figure 3B). What permits  
424 selective LD localization based on AH hydrophobic moment and hydrophobicity is still  
425 unclear. Strong hydrophobicity clearly promotes LD binding but specificity between LDs and  
426 bilayers is then lost [85]. Thus, there might exist a range of optimal hydrophobicity and  
427 hydrophobic moment that enables LD binding specificity.

428

#### 429 **4.5. Membrane properties affecting protein recruitment to LDs**

430 Early lipidomics studies suggest that the ER membrane is asymmetric in its phospholipid  
431 composition [86, 87]. Since LDs emerge from the cytosolic leaflet of the ER membrane, the  
432 LD phospholipid monolayer might be similar to that of the ER cytosolic leaflet. However,  
433 specific enzymes may edit the LD phospholipidome during LD formation making it different  
434 from ER-composing monolayers [6, 28]. Such editing processes might be necessary for  
435 controlling LD size and protein content.

436 The LD surface differs from a bilayer membrane by many biophysical parameters  
437 including hydrophobicity, polarity, lipid packing and thickness (Figure 3D). A phospholipid  
438 monolayer is not static. Phospholipids are diffusive and can temporally cluster, which  
439 provokes transient exposure of the hydrophobic neutral lipids to the aqueous phase [85].  
440 Depending on the AH amino acid composition, class II protein binding can be initiated on  
441 these transient packing defects, i.e. space available between phospholipids. For instance,  
442 molecular dynamics simulations have shown that bulky hydrophobic residues initiate the  
443 binding of the AH M-domain of CCT $\alpha$  on these defects and trigger AH folding at the interface  
444 [85] (Figure 3E). The rate, frequency, and size of these defects very likely depend on the  
445 phospholipid packing level, shape, and neutral lipids. Many AHs selectively bind to LDs and  
446 not to bilayers. If lipid packing defects are the driving force for AH binding, then for an  
447 identical phospholipid composition, binding to LDs should be more favorable for an AH [65]  
448 because of the existence of a larger packing defect on the LD monolayer. In fact, for LDs that  
449 are contiguous with a bilayer membrane, which can serve as a phospholipid reservoir, the  
450 phospholipid distribution between the bilayer and the monolayer membrane is not identical:  
451 for 187 phospholipids to be shared between one leaflet of the bilayer membrane and the  
452 monolayer of the LD, 100 would be on the bilayer side and 87 on the droplet side. In other  
453 words, the monolayer on the LD is 13% less packed than the leaflet of the bilayer (accepted  
454 manuscript: DOI: 10.1083/jcb.201907099). Consistently, *in silico* studies support the  
455 occurrence of more lipid packing defects in a phospholipid bilayer sandwiching an oil layer  
456 than a pure bilayer [85, 88]. Based on these observations, large packing defects would  
457 appear more frequently on LDs and consequently the “on rate” of AHs would be higher on  
458 LDs (Figure 3F). A modulator of the phospholipid monolayer density of ER-detached LDs is

459 the Arf1/COPI machinery. COPI buds nano-droplets from artificial and purified micrometric  
460 LDs, thereby depleting phospholipids from the donor LD and promoting the recruitment of  
461 AHs to this LD [32, 33]. Likewise, seipin (Fld1), in concert with Ldb16 in yeast, may also  
462 control the phospholipid density on LDs since their deletion cause the recruitment of proteins  
463 bearing AHs featured with lipid packing sensing motifs, such as Kes1, to LDs [89].  
464 Potentially, by interacting with anionic phospholipids [28], seipin is capable to edit the  
465 phospholipidome of LDs and subsequently to alter the binding spectrum of AHs.

466 Besides phospholipid packing and composition, other differences exist between a  
467 bilayer and a monolayer: The neutral lipid core of LDs is different from the hydrophobic core  
468 of a bilayer that consists of phospholipid acyl chains. This difference in hydrophobicity can be  
469 sensed by AHs since the nature of the hydrophobic phospholipid packing defect determines  
470 the recruitment level of an AH (accepted manuscript: DOI: 10.1083/jcb.201907099).  
471 Furthermore, membrane thickness plays a crucial role in determining the localization of  
472 protein transmembrane domains [74]. The hydrophobic thickness in the limiting LD  
473 monolayer is infinite at a protein scale, while it is ~3 nm for a bilayer membrane [90] (Figure  
474 3D). As stated above, this difference in thickness might play a role particularly in class I  
475 protein partitioning between ER and LDs. Finally, bilayer membranes are permeable to water  
476 molecules while the thick hydrophobic core of LDs will *a priori* tolerate much less the  
477 presence of water molecules. These are fundamental topological differences that can result  
478 in divergences in the energy landscape of the interaction between a protein domain and a  
479 bilayer and a LD monolayer, respectively. Such differences might control the preferential  
480 binding of a protein between a bilayer and a monolayer (Figure 3G).

481 The basic principle of protein binding is the adoption of a conformation that minimizes  
482 energy at a membrane interface. This is mostly achieved by exposing hydrophobic residues  
483 to the bilayer hydrophobic region and hydrophilic ones to the aqueous phase, thereby  
484 minimizing overall stress in the system (Figure 3E). Binding can preferentially occur to  
485 membranes with specific lipid composition, compressibility, or thickness [91]. These  
486 parameters are extremely changed in the case of LDs and could determine the binding-  
487 selectivity of proteins to LDs. For instance an HP domain in a bilayer interacts with  
488 phospholipid acyl chains and water molecules that can cross the bilayer. On LDs, such  
489 domains would interact primarily with neutral lipids and potentially phospholipid acyl chains,  
490 depending on the monolayer packing level. Thus, because LDs offer a more hydrophobic  
491 environment, it could be that an HP, dipped into the oil phase, favorably accumulates to LDs.  
492 Such recruitment to LDs will concomitantly alleviate ER bilayer stress caused by unfavorable  
493 interactions of the HP with the phospholipid bilayer environment [91] (Figure 3G).

494 The oil/water interface of LDs is of high energy cost and recruiting any amphipathic  
495 molecule will decrease this energy. Thus, the LD surface would be permissive to the binding

496 of AHs but cells have set up diverse regulatory levers to prevent non-selective recruitment.  
497 Therefore, it is not a single parameter that permits a selective AH recruitment to LDs but  
498 rather a concerted action of the regulatory levers. For example, the nature of the interaction  
499 of an AH with the neutral lipid content of LDs is crucial for recruitment. The phospholipid  
500 packing then regulates the amount of neutral lipid accessible to AHs but AHs with high  
501 binding strengths, especially those containing bulky hydrophobic residues, can be non-  
502 specifically recruited (accepted manuscript: DOI: 10.1083/jcb.201907099) [85]. Non-specific  
503 binding is abolished by macromolecular crowding by LD surface gatekeepers such as the  
504 perilipins [75, 92]. PLIN1 for instance binds strongly to LDs and crowds out other proteins  
505 that could non-specifically bind to LDs [75, 92]. This is not the case for PLIN2 and PLIN3,  
506 which are weaker binders and can be displaced from the LD surface by other proteins [75]. In  
507 plant oil bodies, oleosins play a similar role as PLIN1. With their long hairpins, they strongly  
508 associate with the LD surface and control its proteome [93]. By controlling the expression  
509 levels of proteins that can strongly associate with the LD surface, cells can fine-tune the LD  
510 proteome by molecular crowding mechanisms. Finally, other mechanisms involving  
511 interaction with phospholipid headgroup charges or with “genuine” LD proteins can promote  
512 specific recruitment to LDs [36, 66].

513

## 514 **5. Future directions / Conclusions**

515 Our knowledge about the biogenesis of LDs including the recruitment of LD proteins has  
516 expanded significantly over the past decade. We are, however, still at the beginning to  
517 mechanistically understand these processes and future challenges include the identification  
518 of the physico-chemical parameters and the regulatory proteins involved. Bioinformatic tools  
519 are in general very reliable for the prediction of secondary structures and membrane  
520 topologies of bitopic and polytopic membrane proteins that are inserted into phospholipid  
521 bilayers. For monotopic proteins as they are located on LDs, however, these predictions are  
522 often not consistent with experimental data. It will be important to expand current algorithms  
523 to parameters that allow the distinction of bilayer- and monolayer integrated proteins *in silico*.  
524 For this, in-depth biochemical and biophysical analyses of monotopic membrane proteins  
525 with regard to the intrinsic features as well as the collective processes that govern correct  
526 protein-lipid interactions are of fundamental importance.

527

528

## 529 **Conflict of interest**

530 The authors declare no conflict of interest.

531

532

533 **Acknowledgements**

534 We are grateful to David Mick for critical reading of the manuscript and constructive  
535 feedback.

536

537 Support is gratefully acknowledged from the Deutsche Forschungsgemeinschaft (DFG,  
538 CRC1027/C09) to B.S., the ANR-NANODROP, ANR-17-CE11-0003 ANR-MOBIL, ANR-18-  
539 CE11-0012-01, and Paris Sciences et Lettres to A.R.T.

540

541 While we attempted a balanced literature review within the scope of this article, we apologize  
542 to all our colleagues whose work could not be cited due to space restrictions.

543

544

545 **Figure legends**

546

547 **Figure 1: LD biogenesis, architecture and associated proteins**

548 Lipid Droplets (LDs) originate from the endoplasmic reticulum (ER), where triglycerides (TG)  
549 are synthesized. Local accumulation of TG eventually leads to the budding of LDs from the  
550 cytoplasmic leaflet of the ER bilayer membrane. They finally consist of a hydrophobic neural  
551 lipid core, which is surrounded by a phospholipid monolayer. Class I LD proteins are initially  
552 inserted into the ER membrane in a monotopic hairpin topology and from there partition to  
553 the LD surface (blue). Class II LD proteins are recruited to the LD surface from the cytosol  
554 and can directly interact with the phospholipid monolayer *via* amphipathic helices or lipid-  
555 anchors, or alternatively associate with the LD surface *via* protein-protein interactions  
556 (green).

557

558 **Figure 2: Targeting of class I proteins to LDs via the ER**

559 A. LD-destined class I proteins are first inserted into the membrane of the endoplasmic  
560 reticulum (ER). For some class I proteins such as the plant oleosins evidence suggests that  
561 they can employ the conventional signal recognition particle (SRP)- mediated ER targeting  
562 pathway (left panel). For most bitopic or polytopic membrane proteins, SRP binds their signal  
563 sequences that are emerging from the translating ribosome and mediates the recruitment of  
564 the ribosome-nascent-chain complex to the ER membrane in a SRP-receptor (SR)-  
565 dependent manner. Co-translational protein translocation is usually facilitated by the Sec61  
566 translocon complex and signal sequences may be cleaved off by signal peptidase (SPase).  
567 Other class I proteins such as UBXD8 are recognized by PEX19 and post-translationally  
568 inserted into distinct ER subdomains in a PEX3-dependent fashion. PEX19 and PEX3 are  
569 also essential for protein targeting to peroxisomes. While the farnesylation of PEX19 is

570 dispensable for peroxisome biogenesis, it is essential for the correct targeting of UBXD8 to  
571 the ER and subsequent partitioning to LDs.

572 B. Regulation of ER-to-LD partitioning of class I proteins: Since bilayer-spanning  
573 transmembrane domains restrict LD localization, a monotopic hairpin topology is probably a  
574 basic criterion to enable bilayer-to-monolayer partitioning. This may be a passive diffusion  
575 mechanism during LD emergence from the ER bilayer or it may be actively controlled by  
576 gatekeeper proteins recruiting specific hairpin proteins. Sequestering hairpin proteins to ER-  
577 resident tethers can restrict the protein population that is free to partition to LDs.

578

579 **Figure 3: Structural aspects of amphipathic helices and differential binding to bilayer-**  
580 **and monolayer membranes**

581 A: Box shows color codes for the different classes of amino acids. Left panel illustrates  
582 typical AHs with high hydrophobic moments, i.e. well-delineated hydrophobic and hydrophilic  
583 faces. Although the hydrophobic moment can be high for different AHs, the overall  
584 hydrophobicity can differ and be increased by bulky hydrophobic residues. Right panel: the  
585 hydrophobic moment is decreased by a non-polarized distribution of hydrophobic and  
586 hydrophilic residues. B: Charges can influence binding. Left, charges at the edge between  
587 hydrophilic and hydrophobic faces can interact with phospholipid headgroups and stabilize  
588 binding. Right, opposite charges on the hydrophilic face can promote lateral AH-AH  
589 interactions and stabilize AH binding. C: Repeating an AH motif conserves hydrophobic  
590 moment and hydrophobic fraction. However, the overall hydrophobicity will be increased,  
591 influencing binding as observed with Plin4. D: Fundamental divergences between a bilayer  
592 and a monolayer that can determine which proteins bind to which surface. Differences are in  
593 the hydrophobic milieu and polarity: water molecules can cross the hydrophobic milieu of a  
594 bilayer, which is established by phospholipid acyl chains. At a LD interface, it is unlikely that  
595 proteins probe the thickness of the neutral lipid phase. A bilayer is about 3 nm in thickness  
596 while that of a monolayer is “infinite”. The phospholipid packing differs likewise between  
597 these membrane interfaces. The packing of a well-packed bilayer can be increased only at  
598 about 5 % while that of a phospholipid monolayer of a LD can be infinitely varied. E: The  
599 hydrophobic side of an AH in a bilayer interacts essentially with phospholipid acyl chains  
600 while on a LD surface it interacts mainly with neutral lipids which are more accessible; these  
601 two types of interactions could significantly differ. F: A droplet-embedded-vesicle approach  
602 reveals that the phospholipid coverage of a LD emerging from a bilayer is about 10% less  
603 than the phospholipid packing of the monolayer leaflet of the bilayer. Consequently, the “on”  
604 rate of AH on LDs can be expected to be higher. G: Hairpins have different interactions in a  
605 bilayer or a LD monolayer environment. Differences in the interaction free energy will impact  
606 on the bilayer-to-monolayer partitioning of hairpins.

607

608

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610

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class I LD proteins

class II LD proteins





