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Valentin Guyard, Vera Filipa Monteiro-Cardoso, Mohyeddine Omrane, Cécile Sauvanet, Audrey Houcine, Claire Boulogne, Kalthoum Ben Mbarek, Nicolas Vitale, Orestis Faklaris, Naima El Khallouki, et al.

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1 **ORP5 AND ORP8 ORCHESTRATE LIPID DROPLET BIOGENESIS AND**  
2 **MAINTENANCE AT ER-MITOCHONDRIA CONTACT SITES**

3

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27        **SUMMARY**

28        Lipid droplets (LDs) are the primary organelles of lipid storage, buffering energy  
29        fluctuations of the cell. They store neutral lipids in their core that is surrounded by a  
30        protein-decorated phospholipid monolayer. LDs arise from the Endoplasmic Reticulum  
31        (ER). The ER-protein seipin, localizing at ER-LD junctions, controls LD nucleation and  
32        growth. However, how LD biogenesis is spatially and temporally coordinated remains  
33        elusive. Here, we show that the lipid transfer proteins ORP5 and ORP8 control LD  
34        biogenesis at Mitochondria-Associated ER Membrane (MAM) subdomains, enriched  
35        in phosphatidic acid. We found that ORP5/8 regulate seipin recruitment to these MAM-  
36        LD contacts, and their loss impairs LD biogenesis. Importantly, the integrity of ER-  
37        mitochondria contact sites is crucial for the ORP5/8 function in regulating seipin-  
38        mediated LD biogenesis. Our study uncovers an unprecedented ORP5/8 role in  
39        orchestrating LD biogenesis at MAMs and brings novel insights into the metabolic  
40        crosstalk between mitochondria, ER, and LDs at membrane contact sites.

41

42        **KEYWORDS**

43        Lipid droplets, organelle biogenesis, membrane contact sites, MAM, ORP, seipin,  
44        phosphatidic acid

45

46        **HIGHLIGHTS**

47        ORP5 and ORP8 localize at MAM subdomains where LDs originate.

48        Phosphatidic acid is enriched in MAM subdomains that are the birthplace of LDs.

49        ORP5 and ORP8 knockdown impairs LD biogenesis.

50        ORP5 and ORP8 regulate seipin recruitment to MAM-LD contact sites.

51

52

53           **INTRODUCTION**

54           Lipid Droplets (LDs) are evolutionarily conserved organelles that play a primary  
55           role in regulating lipid metabolism by storing lipids in excess and releasing them upon  
56           cellular needs (Olzmann and Carvalho, 2019). They store neutral lipids in their core,  
57           mainly triacylglycerol (TG) and sterol esters, surrounded by a protein-decorated  
58           phospholipid monolayer (Thiam et al., 2013). The storage of these lipids is essential  
59           for the cells to respond to energy fluctuations. Yet, LDs are also involved in other  
60           cellular functions such as protein degradation, gene expression regulation, lipid  
61           sequestration, and membrane biosynthesis (Welte and Gould, 2017).

62           LDs arise from the Endoplasmic Reticulum (ER) following the biosynthesis and  
63           deposition of neutral lipids in the bilayer hydrophobic region (Thiam and Ikonen, 2020).  
64           The neutral lipids condense to nucleate a nascent LD, which grows and buds into a  
65           mature LD. Defects in LD biogenesis and regulation are the hallmark of multiple  
66           metabolic and non-metabolic disorders such as type II diabetes, heart diseases, or  
67           viral infections (Gluchowski et al., 2017; Herker et al., 2021).

68           Seipin is a conserved ER protein forming a large oligomeric ring complex (Klug et  
69           al., 2021; Sui et al., 2018; Yan et al., 2018): it is a master regulator of LD biogenesis,  
70           and it plays a critical role in adipogenesis (Rao and Goodman, 2021). Mutations in  
71           seipin result in impaired lipid and calcium metabolism (Bi et al., 2014; Pagac et al.,  
72           2016) and cause lipodystrophies and neuronal disorders (Magré et al., 2001; Rao and  
73           Goodman, 2021; Windpassinger et al., 2004). Seipin physically marks the site of LD  
74           nucleation and mediates LD growth (Chung et al., 2019; Fei et al., 2008; Salo et al.,  
75           2019; Szymanski et al., 2007). It also regulates the physical connection of newly  
76           formed LDs to the ER, at ER-LD contact sites (Schuldiner and Bohnert, 2017), and  
77           facilitates LD growth (Choudhary et al., 2020; Grippa et al., 2015; Salo and Ikonen,

78 2019; Salo et al., 2019; Wang et al., 2016). In human cells, the oligomeric seipin ring  
79 consists of 11 subunits that bind negatively charged phospholipids, *in vitro*, including  
80 phosphatidic acid (PA) (Yan et al., 2018). In the absence of seipin, PA levels likely  
81 increase in the ER membrane (Fei et al., 2011; Han et al., 2015; Wolinski et al., 2015).  
82 Interestingly, seipin deletion leads to PA accumulation into the inner nuclear envelope  
83 and the formation of nuclear LDs (Sollitsy et al., 2021). These observations support  
84 the hypothesis that seipin is recruited to ER subdomains, probably enriched in  
85 negatively charged lipids (especially PA), where it could form a scaffold that assists LD  
86 assembly.

87 The existence of ER-subdomains promoting LD biogenesis is supported by several  
88 observations (Choudhary et al., 2020; Hariri et al., 2018; Joshi et al., 2018; Santinho  
89 et al., 2020; Wang et al., 2018). LD biogenesis can occur at the ER contact sites with  
90 vacuoles in yeast (Hariri et al., 2018) or peroxisomes in yeast and mammals (Joshi et  
91 al., 2018, 2021). Based on these observations, contact sites established between the  
92 ER and other organelles could play a critical role in LD formation. For example, such  
93 contact sites may pool key enzymes and lipid intermediates necessary for LD  
94 assembly (Choudhary et al., 2020) or preset optimal local physical properties for the  
95 neutral lipids' condensation into LDs. Whether LD can originate at other inter-organelle  
96 contact sites is unknown.

97 Perturbations of the lipid composition in ER are detrimental to proper LD formation  
98 (Adeyo et al., 2011; Ben M'barek et al., 2017; Fei et al., 2011; Zoni et al., 2021).  
99 Therefore, locally editing the ER phospholipid composition, possibly by lipid transfer at  
100 ER-LD contact sites, could be essential for LD biogenesis. However, the existence of  
101 such a mechanism is currently unknown. Also, it is still elusive whether and how lipids  
102 are directly transferred from the ER to the LDs during and after LD formation.

103 Therefore, mechanisms that spatially and temporally regulate LD biogenesis in the cell  
104 remain poorly understood.

105 The Oxysterol binding protein (OSBP)-related proteins constitute a large family  
106 of lipid transfer proteins (LTPs) conserved from yeast (Osh) to humans (ORP) and  
107 localized to different subcellular sites, shown in several cases to be membrane contact  
108 sites. A common feature of all ORPs is the presence of an OSBP-related lipid-  
109 binding/transfer (ORD) domain. Most ORP proteins contain a two phenylalanines (FF)  
110 in an acidic tract (FFAT)-motif that binds ER-localized VAP proteins and a pleckstrin  
111 homology (PH) domain that interacts with lipids or proteins in distinct non-ER organelle  
112 membranes. Two members of this family, ORP5 and ORP8, do not contain an FFAT  
113 motif but are directly anchored to the ER through a C-terminal transmembrane (TM)  
114 segment.

115 ORP5 and ORP8 have been originally shown to localize at ER-PM contact sites  
116 where they transfer phosphatidylserine (PS) from the cortical ER to the PM, in counter-  
117 exchange with the phosphoinositides Phosphatidylinositol-4-phosphate (PtdIns(4)P)  
118 and Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P) (Chung et al., 2015; Ghai et  
119 al., 2017). Lately, ORP5 has been shown to also localize at ER-LD contact sites (Du  
120 et al., 2020) and, although an experimental demonstration is still missing, it has been  
121 proposed that, at these sites, ORP5 could also act as PS/PtdIns(4)P lipid exchanger.

122 We recently showed that ORP5 and ORP8 are also localized, and even enriched,  
123 at a specific ER subdomain in contact with mitochondria, the so-called mitochondria-  
124 associated membranes (MAM), where they mediate transfer of PS, maintain  
125 mitochondrial morphology and function, and control calcium fluxes (Galmes et al.,  
126 2016; Rochin et al., 2019). We hypothesized that MAMs containing ORP5 and ORP8  
127 are involved in LD biogenesis.

128           Here, we show that ORP5 and ORP8 localize at MAM subdomains where LDs  
129 originate and that are enriched in PA lipid. Oleic acid treatment leads to massive  
130 recruitment of ORP5-labeled MAM to nascent and pre-existent LDs, suggesting a dual  
131 role in LD biogenesis and maintenance/turnover. We also reveal that ORP5 and ORP8  
132 are novel players involved in LD biogenesis by regulating seipin recruitment to MAM-  
133 LD contacts.

134

135

136 **RESULTS:**

137 **ORP5 localize at MAM subdomains closely associated with LDs**

138 ORP5 and ORP8 were originally identified as components of ER-PM contact sites  
139 (Chung et al., 2015). They have been recently shown to also localize at contact sites  
140 between ER and mitochondria (Mito) (Galmes et al., 2016) and to primarily interact at  
141 MAMs (Rochin et al., 2021). Lately, a novel localization at ER-LD contact sites upon  
142 oleic acid (OA) treatment has been described for the two ORP5 isoforms (ORP5A and  
143 ORP5B) but not for ORP8 (Du et al., 2020). However, ORP5 distribution at the multiple  
144 contact sites, especially upon OA, remains controversial. Also, whether ORP8 could  
145 also localize at ER-LDs contact sites is unknown.

146 Thus, we first investigated the localization of EGFP-tagged ORP5A, ORP5B (a  
147 natural variant of ORP5, lacking a large part of the PH domain), and ORP5 $\Delta$ PH (a  
148 variant completely lacking its PH domain) by confocal microscopy in HeLa cells treated  
149 for 2h with OA or untreated. LipidTox (LTox) was used to stain LDs and mitotracker to  
150 label mitochondria (Fig 1A-B, S1A).

151 EGFP-ORP5A was detected at cortical ER (ER-PM contacts) in all cells analyzed  
152 (OA treated and untreated), while only a subset of cells displayed ORP5A localization  
153 at ER-LD contacts (20% for untreated and 40% for OA-treated cells). Interestingly, the  
154 ORP5 labeled-ER in contact with LDs appeared to be highly expanded as it almost  
155 completely surrounded the LD surface. EGFP-ORP5B was instead detected at  
156 reticular ER (due to the loss of PM binding) in all cells analyzed and at expanded ER-  
157 LD contact sites in 22% of untreated cells and in about 60% of OA-treated cells.  
158 Similarly, ORP5 $\Delta$ PH was found at ER-LDs contact sites. However, the number of cells  
159 showing ORP5-labeled ER in contact with LD was considerably higher in the pool of



160 cells transfected with ORP5 $\Delta$ PH (80% for untreated and 96% for OA-treated cells) than  
161 in the pool of cells transfected with EGFP-ORP5B (Fig 1B and S1A).

162 Interestingly, almost all the ER subdomains in contact with LDs where ORP5A and  
163 ORP5B localized were found closely associated to mitochondria, forming a novel  
164 three-way Mito-MAM-LD membrane contact site structure (Fig 1A). Quantification of  
165 these observations revealed that 60% of EGFP-ORP5A-, 80% of EGFP-ORP5B- and  
166 80% EGFP-ORP5 $\Delta$ PH-positive ER structures corresponded to Mito-MAM-LD contact  
167 sites, while 35% of EGFP-ORP5A-, 15% of EGFP-ORP5B- and 15% EGFP-  
168 ORP5 $\Delta$ PH-positive ER structures corresponded to MAM. Only a small pool of EGFP-  
169 ORP5A-, of EGFP-ORP5B- and EGFP-ORP5 $\Delta$ PH-positive ER structures (4%, 4%,  
170 and 3%, respectively) were associated only to LDs (Fig 1C).

171 To better visualize the three organelles' association, we proceeded by cell  
172 swelling. Cells were subjected to OA for 2hr and then to a hypotonic medium, which  
173 swells the bilayer-surrounded organelles only. We found that 70% of LDs formed were  
174 in close association with mitochondria and ER vesicles; the remaining 30% were in  
175 contact with ER only (Fig S1B). This observation confirmed the development of Mito-  
176 MAM-LD contact sites upon OA addition. Interestingly, the pool of ORP5 at the MAM-  
177 LD contact was 15 times higher than in the ER regions, indicating that OA addition  
178 massively relocalizes ORP5B from the ER to MAM subdomains in contact with LDs.

179 Furthermore, super-resolution Structured Illuminated Microscopy (SIM) analysis of  
180 EGFP-ORP5A and EGFP-ORP5 $\Delta$ PH localization in HeLa cells treated for 2h with OA  
181 confirmed their localization at Mito-MAM-LD close associations (Fig 1D).

182 To further study these three-way Mito-MAM-LD associations, we assessed their  
183 morphology through ultrastructural analysis by HRP-KDEL EM (carrying a horseradish  
184 peroxidase (HRP) tagged with an ER retention motif to stain the ER) in cells expressing

185 HRP-KDEL alone or together with EGFP-ORP5A or EGFP-ORP5 $\Delta$ PH (Fig 1E, Fig  
186 S1C). Our morphological analysis confirmed that the Mito-MAM-LD associations  
187 observed by light microscopy were indeed membrane contact sites (in the range of 10-  
188 30nm). Agreeing with our confocal data, ORP5A, and ORP5 $\Delta$ PH to a greater extent,  
189 induced an expansion of the ER on the LD surface. Mito-MAM-LD associations were  
190 also detected in cells expressing HRP-KDEL alone, revealing the existence of this  
191 structure even in the absence of ORP5 overexpression. However, the ER involved in  
192 these contact sites was not highly-expanded around the LDs (Fig 1E, Fig S1C),  
193 possibly explaining why these associations have been overlooked so far.

194 Moreover, the localization of ORP5 at the expanded Mito-MAM-LD contact sites  
195 was confirmed by immunogold labeling on ultrathin cryosections of cells expressing  
196 ORP5 $\Delta$ PH and treated with OA. The advantage of using ORP5 $\Delta$ PH over ORP5A or  
197 ORP5B relies on the higher number of cells showing its localization at Mito-MAM-LD  
198 contact sites (see Fig 1B), facilitating the immuno-EM analysis (Fig 1F). The bulk of  
199 ORP5 $\Delta$ PH localized to Mito-MAM-LD contact sites and to the associated ER-LD  
200 contacts, while a minor pool was detected at MAM (ER-mitochondria contacts) not  
201 associated with LDs (Fig 1F).

202 Together, these data reveal the existence of a novel tripartite Mito-MAM-LD  
203 contact sites junction where ORP5 localizes. However, it is not known whether its  
204 binding partner ORP8 also localizes at Mito-MAM-LD contact sites.

205

### 206 **ORP8 is enrichment at MAM-LD via interactions with ORP5 by its coiled-coil** 207 **domain**

208 We overexpressed EGFP-ORP8 and analyzed its localization by confocal  
209 microscopy in HeLa cells treated with OA (Fig 2A). In contrast to ORP5, ORP8 mostly

210 localized to the reticular ER, with a minor pool additionally present at cortical ER and  
211 MAMs, as previously shown (Galmes et al., 2016; Rochin L, 2020). A small pool of  
212 ORP8 was detected at MAM-LD contact sites, but the ER elements labeled with ORP8  
213 were not expanded around the LD surface as seen for ORP5.

214 ORP5 and ORP8 interact at MAMs (Rochin et al., 2021). We thus reasoned that  
215 the faint signal of ORP8 detected at the MAM-LD contact in overexpression conditions  
216 could be due to a lack of ORP5. We then overexpressed EGFP-ORP8 with RFP-  
217 ORP5B. Under this condition, ORP8 dramatically redistributes from the reticular and  
218 cortical ER to the ORP5-positive MAM-LD contact sites. This redistribution and  
219 enrichment at MAM-LD contacts were not observed in the case of a general ER protein  
220 such as Sec61b, which, even when co-expressed with ORP5, maintained its  
221 localization to the reticular ER (Fig S2A). These findings confirmed the specificity of  
222 ORP8 localization at MAM-LDs. Altogether, these data indicate that ORP5 has a higher  
223 affinity for MAM-LD contacts than ORP8, and that ORP8 enrichment at such sites  
224 depends on its interaction with and on the expression level of ORP5.

225 Deletion of the ORD domain from ORP5 eliminates its ability to localize at MAM-  
226 LDs (Fig S2B). This is in accord with recent data showing that the ORD domain of  
227 ORP5 is required for its localization to ER-LDs contacts (Du et al., 2020). How and  
228 whether this domain in ORP5/8 directly bind to LD surfaces is unknown. Thus, we  
229 decided to test *in vitro* whether these cytosolic ORD domains can interact with the  
230 neutral lipid/water interface of LDs and to compare their binding affinities. We used a  
231 tensiometer approach where we generated a triolein-in-buffer droplet (Ajjaji et al.,  
232 2019). The tension of this interface was 34mN/m, and then we added either the ORD  
233 domain of ORP5 or ORP8 at the same concentration. Adsorption of these domains to  
234 the interface will decrease tension; the better the adsorption, the higher the decrease

235 in tension. We found that the ORD domain of ORP5 systematically decreased much  
236 better tension, from 34 to 18mN/m, than of ORP8, from 34mN/m to 20mN/m (Fig 2B).  
237 These experiments revealed that both ORD5 and ORD8 could bind LDs, but the  
238 binding ability of ORD8 would be lower than ORD5. Expression of EGFP-tagged ORDs  
239 in HeLa cells confirmed that the ORD domain of ORP5 (EGFP-ORD5) strongly binds  
240 LDs, while the ORD domain of ORP8 (EGFP-ORD8) only weakly binds LDs (arrows,  
241 Fig 2C). These data might explain why ORP8 overexpression does not induce an  
242 expansion of ER around the LDs and also suggest that ORP8 requires ORP5 to be  
243 enriched at the expanded MAM-LDs contact sites.

244 To more directly test whether ORP8 binding to LDs requires ORP5 and the  
245 domains involved, we co-expressed an ORP5 deletion mutant lacking the ER-  
246 anchoring TM domain (RFP-ORP5 $\Delta$ TM) with an ORP8 construct carrying a similar  
247 mutation (EGFP-ORP8 $\Delta$ TM) or with ORP8 lacking both the TM and the PH or the  
248 coiled-coil (CC) domains (EGFP-ORP8- $\Delta$ PH $\Delta$ TM or EGFP-ORP8- $\Delta$ CC $\Delta$ TM) and  
249 analyzed their recruitment to LDs in HeLa cells by confocal microscopy (Fig 2D). The  
250 EGFP-ORP5 $\Delta$ TM, when expressed alone, localized to both the PM and LDs, while the  
251 EGFP-ORP8 $\Delta$ TM mostly localized to the cytosol, to the PM, and weakly to LDs (Fig  
252 S2C). However, when co-expressed with RFP-ORP5 $\Delta$ TM, EGFP-ORP8 $\Delta$ TM strongly  
253 co-localizes with ORP5, as assessed by the Pearson's correlation coefficient analysis,  
254 and enriches at the ORP5-labeled LDs (Fig 2D-E). Of note, ORP5 $\Delta$ TM and ORP8 $\Delta$ TM,  
255 when co-expressed, lose their PM localization and strongly redistribute to LDs, and  
256 also to cytosolic filamentous structures that resemble cytoskeleton.

257 The concomitant deletion of the PH domain in ORP8 $\Delta$ TM does not induce  
258 significant changes in ORP8 colocalization with ORP5 at LDs. However, the deletion  
259 of the CC domain in ORP8 $\Delta$ TM completely abolished ORP8 targeting to the ORP5-

260 labeled LDs (Fig 2D-E). These results confirm that interaction with ORP5 via the CC  
261 domain is required for the ORP8 binding to LDs.

262 Finally, we determined whether ORP5 and ORP8 interaction and localization at  
263 MAM-LD contacts also occur at endogenous levels. We performed Proximity Ligation  
264 Assay (PLA) by duolink in HeLa cells transfected with Mito-BFP, to stain the  
265 mitochondria and with mCherry-Perilipin 1 (Plin1), to stain the LDs, and analyzed by  
266 confocal microscopy. ORP5-ORP8 PLA spots were detected in the proximity of a  
267 subset of mitochondria-associated LDs (arrows, Fig 2F). To confirm that these sites  
268 corresponded to MAMs, we performed PLA using antibodies against either ORP5 or  
269 ORP8 and their mitochondrial binding partner PTPIP51 (Galmes et al., 2016). A similar  
270 pool of ORP8-PTPIP51 or ORP5-PTPIP51 PLA spots was observed at MAM-LD  
271 contacts (arrows, Fig 2G-H), confirming that ORP5-8 localize and interact at the  
272 tripartite Mito-MAM-LD contact sites in endogenous conditions.

273

#### 274 **ORP5 and ORP8 organize LD biogenesis at MAM**

275 To assess the role of ORP5 and ORP8 in LD biogenesis, we depleted ORP5 and  
276 ORP8 by RNAi in HeLa cells that have been delipidated for 3 days to remove pre-  
277 existent LDs. The efficiency of the knockdown (KD) was validated by western blot (WB)  
278 (Fig S3A-B). LD biogenesis was induced by OA treatment, and cells were imaged by  
279 confocal microscopy at different times (15min, 30min, 1hr, 2hr) (Fig S3A). The  
280 abundance of LDs, stained by LTox, in both ORP5 and ORP8 KD cells was significantly  
281 reduced at all time points, as compared with control (Ctrl) cells. The decrease in LD  
282 number was greater at earlier times (70% at 15 min, 60-65% at 30 min and 1hr, 30-  
283 40% at 2hr), suggesting a delay in LD biogenesis (Fig S3A, S3C). Since LTox may  
284 have marked pre-existing LDs that could have resisted delipidation, we performed

285 parallel experiments by feeding cells micelles containing a fluorescent C<sub>12</sub>-fatty acid  
286 (referred to as FA<sup>568</sup>, associated with red fluorescence), as in (Khaldoun et al., 2014).  
287 This option enabled us to track efficiently the biogenesis and the maturation of newly  
288 synthesized LDs. The efficiency of delipidation was confirmed by the almost complete  
289 disappearance of LTox-positive LDs in Ctrl, ORP5, and ORP8 KD cells at time=0 min  
290 (Fig 3A, 3C). The FA<sup>568</sup> treatment induced the formation of new LDs that were also  
291 labeled by LTox. The number of the FA<sup>568</sup>-containing LDs was dramatically reduced  
292 at all time points (86% at 15 min, 92% at 30 min, and 71% at 1hr) in ORP5 and ORP8  
293 KD cells as compared with control cells. The decrease in newly-formed FA<sup>568</sup>-positive  
294 LDs was higher than the decrease in LTox-positive LDs, which likely included the pre-  
295 existent ones (Fig 3B-C). These results indicate a role of ORP5 and ORP8 in regulating  
296 LD biogenesis.

297 To analyze the morphology of nascent LDs at MAMs, we performed EM analysis  
298 on control and ORP5 KD cells treated with OA. Importantly, we detected specific ER  
299 subdomains that appeared as peculiar electron-dense structures associated with some  
300 LDs connected to tubular ER elements, and that likely correspond to the sites where  
301 these LD emerged from the ER. These ER subdomains very often corresponded to  
302 MAM in control cells (Fig 3C). Quantifications of these EM observations revealed that  
303 ORP5 KD induces a strong decrease in the occurrence of these MAM-emerged LDs  
304 while the ER-emerged LD connections, not in close contact with mitochondria, were  
305 instead increased (Fig 3D). These data strongly suggest that ORP5 regulates LD  
306 formation from the MAM subdomain.

307 We then tested if re-expression of ORP5 could rescue the LD phenotype of the  
308 ORP5 KD cells. To this purpose, we re-expressed siRNA-resistant EGFP-ORP5B or  
309 EGFP-ORP5 $\Delta$ PH or, as a control, the ER marker protein EGFP-Sec61 $\beta$  in the

310 delipidated ORP5 KD cells. To monitor rescue of the LD phenotype, we performed  
311 FA<sup>568</sup>-mediated induction of LD biogenesis, as described above, and analyzed by  
312 confocal microscopy the number of FA<sup>568</sup>-positive LDs at 15 minutes after its delivery.  
313 Remarkably, both EGFP-ORP5B and EGFP-ORP5 $\Delta$ PH constructs significantly  
314 rescued the LD decrease in siORP5 cells, while EGFP-Sec61 $\beta$  was not able to rescue  
315 the LD phenotype (Fig 3E-F, S3E). These results provide a causal relationship  
316 between the lack of ORP5 and the perturbation of LD formation and implicate ORP5B  
317 in the phenotype.

318 To address whether the ER-Mito contact sites could play a role in LD formation,  
319 we decided to perform similar experiments in cells where these contacts are disrupted.  
320 PTPIP51 overexpression increases ER-Mito contact sites, while its knockdown  
321 significantly reduces these contacts (De Vos et al., 2012; Stoica et al., 2014) We  
322 performed PTPIP51 KD in cells delipidated for 3 days (Fig S4A) and loaded the cells  
323 with FA<sup>568</sup> for 15 min to trigger the formation of LDs. We found a dramatic decrease in  
324 the number of FA<sup>568</sup>-positive LDs in the PTPIP51 depleted cells (more than 90%) as  
325 compared to control cells (Fig S4B-C).

326 To test whether ORP5 requires intact ER-Mito contacts to regulate LD biogenesis,  
327 we decided to test whether ORP5 overexpression could compensate for the loss of  
328 PTPIP51. We performed a rescue experiment in cells depleted for PTPIP51 by  
329 overexpressing EGFP-ORP5B and using EGFP-Sec61 $\beta$  as a control. Cells were  
330 delipidated for 3 days, loaded with FA<sup>568</sup> for 1h, and analyzed by confocal microscopy  
331 (Fig 3G). Remarkably, ORP5B overexpression, similarly to EGFP-Sec61 $\beta$ , could not  
332 rescue the decrease in LDs observed upon PTPIP51 depletion (Fig 3G-H, Fig S4D).  
333 These data indicate that the maintenance of the integrity of ER-Mito contact sites is

334 required to ensure ORP5/8-dependent proper LD formation and uncover a novel role  
335 of MAMs as key hotspots for LD formation.

336

337 **ORP5 is recruited to LDs emerging from MAM subdomains and to pre-existing**  
338 **LDs**

339 To characterize the dynamics of ORP5 localization at MAM-LD contact sites, we  
340 performed confocal live-cell imaging by spinning disk microscopy in HeLa cells. Cells  
341 transfected with EGFP-ORP5B and Mito-BFP were treated with FA<sup>568</sup> (at 2 min) and  
342 imaged for 12 min. Immediately after the addition of FA<sup>568</sup>, ORP5B was recruited to  
343 MAM subdomains where de-novo LDs formed and became visible within 8 minutes  
344 following the fatty acid addition (Fig 4A-B, Fig S5A, and movie S1-2).

345 To corroborate these data in another cell model relevant for LD physiology, we  
346 examined the dynamics of ORP5 recruitment at MAM-LDs contact sites in human  
347 hepatocytes Huh7 by Airyscan microscopy in live cells (Airyscan). Huh7 cells were  
348 transfected with EGFP-ORP5B, RFP-Sec22b, and Mito-BFP (Figure 4C), or EGFP-  
349 ORP5B, TOM20-mCherry, and Mito-BFP (Figure 4F). We then imaged a cell and  
350 added OA for 1hr to induce TG synthesis and de novo LD formation.

351 At 20-25 minutes from OA addition, ORP5 started to be enriched in ER  
352 subdomains often corresponding to MAMs, in contact with both LDs and mitochondria  
353 (Figure S5B-S5C). The ER-protein Sec22b also localized at the MAM where ORP5  
354 was recruited (Figure S5B), confirming that these structures were indeed ER. Even  
355 after 1hr from the induction of LD formation, Sec22b maintained its reticular  
356 localization, but it was not enriched at MAM (Fig S5C). In contrast, at 50min-1hr from  
357 the OA addition, ORP5 was still strongly at MAM-LDs contact sites (Fig S5B, S5C-D).



358 OA triggered the strong redistribution of ORP5 from the reticular ER to MAM-LD  
359 contact regions (Figure 4C-4D, S5B, S5C-D) and the fraction of LDs that are positive  
360 for ORP5 increased during feeding (Figure 4D). Tiny LDs, newly emerging, had a  
361 strong ORP5 signal (Figure 4E), close to mitochondria also. This observation is  
362 consistent with the early recruitment of ORP5 at the MAM sites where LDs assembled  
363 in the HeLa cells (Figure 4A, S5A). Thus, both HeLa and Huh7 hepatocyte cells  
364 indicate that ORP5 is involved in orchestrating the early stage of LD assembly.  
365 However, OA treatment induced targeting of ORP5 not only to mitochondria-  
366 associated newly-formed tiny LDs, but also to the preexistent larger ones (Figure 4C-  
367 4F, S5B-C), suggesting a role of ORP5 in LD biogenesis but also in their maintenance  
368 at MAMs, possibly by regulating lipid fluxes toward them to fit local cellular needs.

369

#### 370 **ORP5 is recruited to PA-enriched MAM subdomains**

371 The ORD domain of ORP5 is involved in LD binding, while its PH domain is not  
372 required (Fig 1 and 2), as previously shown (Du et al., 2020). The CC domain, which is  
373 poorly characterized and sits just before the PH domain, has been proposed to play a role  
374 in the association of ORP5 with the plasma membrane (Ghai et al., 2017). We asked  
375 whether the ORP5 CC domain could also be relevant for the targeting of MAM-LDs and  
376 LD binding. To answer this question, we analyzed by confocal microscopy the localization  
377 of the EGFP-tagged ORP5 deletion mutant lacking the CC domain (aa 96-116) (EGFP-  
378 ORP5 $\Delta$ CC) in HeLa cells treated with OA (Fig 5A). As awaited, the deletion of CC in  
379 ORP5A led to the loss of ER-PM contacts localization, indicating that the CC might be  
380 involved in the binding of PM proteins or lipids. EGFP-ORP5 $\Delta$ CC localized to the reticular  
381 ER in all transfected cells and, only in very few cells (about 10%), it was detected at MAM-  
382 LDs contact sites. Also, in these cells, even after OA treatment, ORP5 was not enriched

383 at MAM-LD contacts (Fig 5A). This data strongly supports that the CC domain plays a key  
384 role in the association of ORPs with MAM/LDs.

385 Because the CC domain bears multiple charges, we hypothesized it might bind to  
386 charged lipids at MAM/LDs. To assess the lipid-binding specificities of the ORPs CC  
387 domains, we performed PIP-strip binding assays. Both ORP5 and ORP8 strongly bound  
388 PtdIns(4)P, but ORP8 showed a higher preference for PtdIns(3)P and PtdIns  
389 bisphosphates (except for PtdIns(3,4)P, not recognized by the ORPs). Interestingly,  
390 ORP5 and ORP8 CCs also bound phosphatidic acid (PA), and, although this binding was  
391 quite weak, it was very specific as no other phospholipids were recognized (Fig 5B).

392 PA is a negatively charged non-bilayer lipid, constantly being made in the ER and  
393 essential for the synthesis of phospholipids and TAG, and consequently for LD biogenesis  
394 (Gao et al., 2019). We next sought to investigate the presence of PA at MAM-LD contact  
395 sites by using a PA-specific probe, the EGFP-tagged Opi1pQ2S-PABD (hereon called  
396 EGFP-Opi1p), an improved version of Opi1 for sensing PA. HeLa cells were co-transfected  
397 with EGFP-Opi1p and either RFP-Sec22b, RFP-ORP5B, or RFP-ORP5 $\Delta$ PH and  
398 analyzed by confocal microscopy. EGFP-Opi1p was detected at RFP-Sec22-positive ER  
399 subdomains, and these were often enriched in proximity to mitochondria, revealing the  
400 existence of a specific PA pool at MAMs (Fig 5C). When co-expressed with RFP-ORP5B,  
401 Opi1p was strongly enriched at the ORP5-marked MAM and MAM-LD contact sites. The  
402 enrichment at the latter contact sites was even clearer when Opi1p was co-expressed with  
403 RFP-ORP5 $\Delta$ PH (Fig 5C-D), which had the highest recruitment to MAM-LD contact (Figure  
404 1B, S1A). These data suggest a functional link between PA and ORP5 at MAMs.

405

406 **Seipin localizes to MAM-LD contacts in an ORP5-dependent manner**

407 Seipin is an integral ER membrane protein playing a central role in determining where  
408 LDs form. Interestingly, seipin binds anionic phospholipids, including PA (Yan et al., 2018).  
409 We asked whether seipin could also localize at MAM to facilitate LD formation in proximity  
410 to mitochondria. To address this, we co-expressed in HeLa cells a YFP-tagged mouse  
411 seipin with Mito-BFP, alone or together with RFP-Sec22b. We then analyzed seipin  
412 localization by confocal microscopy (Fig 6A). As expected, seipin colocalized with the ER  
413 protein Sec22b. However, a subset of cells where seipin was expressed at lower levels  
414 displayed additional enrichments of seipin to ER structures (MAM) in close proximity to  
415 mitochondria, which were often also associated with LDs (MAM-LDs). We then asked  
416 whether ORP5 could regulate the localization of seipin at MAM. To address this, in parallel  
417 experiments, we co-expressed YFP-seipin with RFP-ORP5 $\Delta$ PH (Fig 6A). Remarkably,  
418 we observed an increase in the population of cells showing the enrichment of seipin at  
419 MAM-LD contacts (Fig S6A-B).

420 Quantification of the distribution of seipin-positive clusters upon segmentation of the  
421 ER, the LD, and the mitochondria by Imaris (Fig 6B) revealed that, when seipin was  
422 expressed alone or with Sec22b, most of these clusters corresponded to MAM (about  
423 78%) and only a few (20%) to the reticular ER. A fraction of the seipin-positive MAM (18%)  
424 was also closely associated with LDs (corresponding to MAM-LD contacts), and only a  
425 minimal amount of seipin-positive ER clusters (2%) was exclusively associated with LDs.  
426 When seipin was co-expressed with ORP5 $\Delta$ PH, we observed a significant increase of  
427 Seipin-positive MAM in contact with LD and a significant decrease of seipin-positive MAMs  
428 not associated with LD (Fig 6B). However, by confocal imaging, we could not analyze the  
429 localization of the entire ER pool of seipin, but only its local enrichment at these “clusters”.

430 To characterize the distribution of the entire seipin pool at a high-resolution level and  
431 to also analyze the ultrastructure of these seipin “clusters”, we performed immuno-EM

432 analysis on ultrathin cryosection (by Tokuyasu method) in HeLa cells expressing YFP-  
433 seipin alone or together with HA-ORP5 $\Delta$ PH, and performed co-immunolabeling of YFP-  
434 seipin (15nm gold) and protein disulfide isomerase (PDI) (10nm gold) to stain the ER (Fig  
435 6C-D, Fig S6C). When expressed alone, seipin was mostly observed in the widely  
436 distributed reticular ER (70%), but a fraction of seipin (12%) was also found at sites of  
437 apposition between MAMs and mitochondria, and a little pool (3%) was detected at MAM-  
438 LD contacts closely associated to mitochondria. Few cells displayed accumulation of  
439 seipin-positive ER elements in contact with mitochondria that presumably correspond to  
440 the “clusters” observed and quantified by confocal. Consistent with our confocal data,  
441 these clusters were always found in close connection with mitochondria (corresponding  
442 to MAM) and sometimes additionally with LD (corresponding to MAM-LD contacts) (Fig  
443 6C and S6C). Moreover, further corroborating our confocal data, co-expression with  
444 ORP5 $\Delta$ PH increased the pool of seipin at MAM-LD contact sites (3% to 24%)  
445 accompanied by an increase of seipin at ER-LD contacts, because of the ORP5-induced  
446 expansion of ER on the LD surface (from 10% to 24%). This increase was also  
447 accompanied by a significant decrease in seipin abundance at the reticular ER not  
448 associated with contact sites (from 70% to 53%) (Fig 6D). Co-localization of seipin and  
449 ORP5 at the expanded MAM-LD contact sites was further confirmed by co-  
450 immunolabeling of YFP-seipin (15nm gold) and ORP5 (10nm gold) (Fig 6F). Seipin  
451 recruitment to ORP5-positive MAM-LD contact sites upon OA was confirmed in Hu7h cells  
452 co-expressing seipin-EGFP, RFP-ORP5B, and Mito-BFP (Fig S6D). Overall, these  
453 results reveal that seipin localizes at MAM-LD contacts in an ORP5-dependent manner.

454

455 **ORP5 role in seipin recruitment to MAM-LD contacts depends on MAM integrity**

456 To further study the role of ORP5, and also of ORP8, in the targeting of seipin to  
457 MAM-LD contact sites, we analyzed the localization of seipin in ORP5 and ORP8 KD  
458 cells, transfected with YFP-seipin and Mito-BFP and stained by LTox (Fig 7A).  
459 Knockdown of ORP5 and ORP8 led to a significant decrease in the cells showing  
460 seipin clusters at MAM-LD (Fig S7A) accompanied by an increase in seipin association  
461 to the reticular ER (Fig 7A-B). Interestingly, in ORP5-depleted cells, we also detected  
462 a general decrease in the seipin pool associated with MAM, while seipin abundance at  
463 ER-LD contacts was unchanged in both ORP5- and ORP8-silenced cells (Fig 7A-B).  
464 These data reveal a key role of ORP5 and ORP8 in regulating the targeting of seipin  
465 to MAM-LD contacts, and a more important role for ORP5 in regulating the general  
466 recruitment of seipin to MAMs.

467 Next, we wanted to rescue the decrease in seipin at MAM-LDs induced by the  
468 loss of ORP5. For this purpose, we chose to overexpress ORP5 $\Delta$ PH in the ORP KD  
469 background. We transfected siRNA-resistant ORP5 $\Delta$ PH, or RFP-Sec22b as a control,  
470 in the ORP5 depleted cells and analyzed seipin localization by confocal microscopy  
471 (Fig 7C). Re-expression of ORP5 $\Delta$ PH (detected by immunofluorescence using an  
472 antibody against ORP5), but not of RFP-Sec22b, completely rescued the levels of  
473 seipin at MAM and even increased the seipin pool at MAM-LD, confirming the  
474 specificity of ORP5 activity in controlling seipin localization to MAMs, including those  
475 associated with LDs (Fig 7C-D, Fig S7B-C).

476 To further confirm these observations, we analyzed the entire seipin pool localization  
477 at a high-resolution level by performing immuno-EM analysis on ultrathin cryosection in  
478 HeLa cells expressing YFP-seipin. We co-immunolabeled YFP-seipin (15nm gold) and  
479 the luminal ER protein PDI (10nm gold) (Fig 7E, Fig S7D). A dramatic decrease of seipin  
480 localized at MAM-LDs (of about 84%) was observed in ORP5-silenced cells. This

481 decrease was accompanied by a decrease of seipin at ER-LDs and also a slight but  
482 statistically significant decrease of seipin at MAMs (Fig 7E). These results further validated  
483 the key role of ORP5 in regulating the levels of seipin at MAM and at MAM-LD contacts.

484 Finally, to assess whether ORP5 requires intact ER-mitochondria contacts to  
485 regulate seipin targeting at MAMs, we depleted PTPIP51, which we had found involved  
486 in LD biogenesis (see Fig 3G-H and S4). We then analyzed seipin localization by  
487 confocal microscopy. PTPIP51 knockdown induced a dramatic decrease (50%) in the  
488 number of cells showing seipin localization at MAM-LD contacts (Fig S7E). Also, seipin  
489 localization at MAM-LD was greatly reduced (50%), and, in a similar proportion, seipin  
490 localization within the reticular ER was increased (Fig 7F-G). Moreover, re-expression  
491 of ORP5 $\Delta$ PH in PTPIP51 depleted cells did not rescue seipin decrease at MAM-LDs,  
492 indicating that intact ER-mitochondria contacts are required for ORP5 function in seipin  
493 recruitment at MAM-LD contacts.

494

## 495 **DISCUSSION**

496 In this study, we found a novel function of the LTPs ORP5 and ORP8 in regulating  
497 LD biogenesis and growth at MAMs. We showed that the ORP5/8 complex localizes  
498 at MAM subdomains enriched in PA lipid and where LDs originate. The loss of ORP5/8  
499 impairs LD biogenesis. We then revealed that ORP5 and ORP8 regulate seipin  
500 recruitment to the newly-identified MAM-LD contact sites and, importantly, that ER-  
501 mitochondria contact sites integrity is required to ensure ORP5/8 function in proper  
502 seipin-mediated LD biogenesis.

503 Amongst all ORP proteins, ORP2 was first identified to localize to LDs to regulate  
504 cellular sterol homeostasis (Hynynen et al., 2009; Jansen et al., 2011). Recently,  
505 ORP5, but not ORP8, was described at ER-LD contacts (Du et al., 2020). These two  
506 proteins have been previously shown to localize at ER-PM and ER-mitochondria  
507 contact sites (Chung et al., 2015; Galmes et al., 2016) and exist as a protein complex,  
508 mainly at MAMs (Rochin et al., 2021). Yet, ORP5 and ORP8 distribution across all  
509 these contact sites remain controversial. Our multiple high-resolution imaging  
510 approaches revealed that ORP5 and ORP8 localize and interact at MAM subdomains  
511 in contact with LDs (Fig 1-2). Moreover, overexpressed ORP5 induced an expansion  
512 of MAMs around LDs due to the strong ability of the ORP5 ORD domain to bind to LDs  
513 compared with the ORP8 ORD domain when expressed alone. The ability of ORP8 to  
514 enrich in MAM-LD contact sites depends on ORP5 levels: ORP8 enrichment in this site  
515 is increased by interaction with ORP5 through its CC domain. These findings highlight  
516 the existence of a novel tripartite junctional interface between mitochondria, MAM, and  
517 LDs where these two LTPs localize. They also provide novel insights on the role of the  
518 CC in ORP5/8, which function was so far poorly understood, in regulating ORP5/8  
519 localization and interaction at MAM-LD contact sites. Interestingly, the knockdown of

520 the ER-mitochondrial tether PTPIP51 leads to a dramatic decrease in newly formed  
521 LDs (Fig S4), thus revealing that disruption of ER-mitochondria contact sites perturbs  
522 LD biogenesis. This alteration in LD biogenesis was not rescued by the re-expression  
523 of ORP5, indicating that ORP5/8 function in LD biogenesis requires intact ER-  
524 mitochondria contacts (Fig 3G-H and S4D). Our findings reveal that LD biogenesis and  
525 growth occur at ER-mitochondria contact sites, and depend on ORP5/8-activity and  
526 the integrity of MAMs.

527 The metabolic crosstalk between LD and mitochondria is well established for lipid  
528 oxidation or storage purposes (Olzmann and Carvalho, 2018; Veliova et al., 2020).  
529 Contact sites between LDs and mitochondria form in response to starvation (Herms et  
530 al., 2015; Rambold et al., 2015; Wang et al., 2011). During starvation-induced  
531 autophagy, DGAT1 (diacylglycerol acyltransferase 1)-dependent LD biogenesis  
532 protects mitochondria function by converting fatty acids into TG stored in LDs, to  
533 prevent the accumulation of toxic lipids in mitochondria (Nguyen et al., 2017). Recent  
534 studies indicate that LD biogenesis can occur at ER subdomains in contact with  
535 catabolic organelles, such as the yeast vacuole or the peroxisomes (Hariri et al., 2018;  
536 Joshi et al., 2018). Interestingly, DGAT2, one of the two diacylglycerol acyltransferase  
537 enzymes converting diacylglycerols into TG, also localizes to MAM (Stone et al., 2009)  
538 and might induce the formation of specific LDs originating from MAMs.

539 Recently, the mitochondrial protein Mitoguardin 2 has been shown to link  
540 Mitochondria, LD and ER, to promote de novo lipogenesis in adipocytes from non-lipid  
541 precursors (Freyre et al., 2019). Also, interesting, in brown adipocytes, a specific  
542 mitochondria subset with restricted dynamics is bound to LDs (Benador et al., 2018).  
543 These peridroplet mitochondria support the growth of LDs by providing ATP molecules  
544 necessary for TG synthesis (Benador et al., 2018). Also, mitochondria are a site of



545 glycerol biosynthesis, through the Krebs cycle, and could provide glycerol precursors  
546 for TG biosynthesis. Based on these knowledge, it may not be surprising that  
547 mitochondria transiently or permanently interact with MAMs to support LD biogenesis  
548 and maintenance, e.g., by providing ATP or glycerol molecules used to synthesize TG.  
549 Currently, molecular mechanisms regulating the functional crosstalk between LD-ER-  
550 Mitochondria organelles remain largely unknown.

551 The ER phospholipid composition is important for LD formation (Ben M'barek et al.,  
552 2017; Thiam and Forêt, 2016; Zoni et al., 2021). ORP5/8 may regulate LD biogenesis by  
553 regulating the phospholipid composition at MAM. ORP5/8 have been shown to counter-  
554 exchange PS and PtdIns(4)P or PtdIns(4,5)P at ER-PM contacts (Chung et al., 2015; Ghai  
555 et al., 2017). Recently, ORP5 has been proposed to play a similar role at ER-LD contact  
556 sites (Du et al., 2020). However, that ORP5/8 systematically counter exchange PS with  
557 PtdIns(4)P is not established. For instance, we recently found that ORP5/8 can transfer  
558 PS from the ER to mitochondria at ER-mitochondria contacts despite the lack of  
559 PtdIns(4)P on mitochondria. This result suggests that ORP5/8 might carry out multiple  
560 lipid transfer activities and that the underlying mechanisms might be different depending  
561 on the local lipid composition. In particular, several pieces of evidence link PA to LD  
562 biogenesis (Gao et al., 2019; Pagac et al., 2016) and, interestingly, seipin can bind to  
563 PA *in vitro* (Yan et al., 2018). Our confocal and immuno-EM analysis uncovers that  
564 seipin localizes at MAM and MAM associated with LDs, in addition to its previously-  
565 reported localization at ER-LD contact sites (Salo and Ikonen, 2019; Salo et al., 2019;  
566 Szymanski et al., 2007; Wang et al., 2016). This seipin localization to MAM is  
567 dependent on ORP5/8, as the knockdown of the latter decreased seipin at MAM-LD  
568 junctions. At the same time, the overexpression of an ORP5 variant enriched at MAM-  
569 LD induced seipin accumulation at these contact sites (Fig 6-7). This impact of ORP5

570 function in seipin recruitment to MAM-LD depends on the integrity of the ER-  
571 mitochondria contacts. These data identify ORP5 and ORP8 as novel critical players  
572 in LD biogenesis by regulating seipin targeting MAMs.

573       Based on the above observation, one intriguing hypothesis is that ORP5/8 could  
574 be involved in PA metabolism at MAMs. ORP5/8 could enrich and cooperate with PA  
575 at MAM sites via their lipid transfer activity, e.g., from Mito-to-ER. Indeed, we found  
576 that the MAM subdomains where ORP5 localize are enriched in PA lipid (Fig 5),  
577 providing the first evidence of the enrichment of PA at MAM. Such acute regulation of  
578 local PA levels at MAMs could be necessary for stabilizing the seipin complex, which  
579 then better controls LD nucleation and growth by fueling TG from the ER to the newly  
580 formed or pre-existing LDs. Such local PA and seipin recruitment by ORP5/8 is here  
581 triggered OA induction, which massively redistributes ORP5 to the MAM subdomains  
582 in contact with both nascent and pre-existent LDs (Fig 4 and S5). As the CC of ORP5/8  
583 binds to PA *in vitro* (Fig 5), it is possible that in addition to being involved in ORP5-8  
584 interaction, the CC domain of ORP5/8 is also involved in their recruitment to the PA-  
585 enriched MAM-LD subdomains, or recruit PA; deletion of the CC decreased ORP5  
586 targeting to MAM-LD contact sites.

587       Finally, our EM analysis unveils the morphological features of the ER subdomains  
588 from which LDs emerge. In cells treated with OA, we observed electron-dense  
589 membrane structures, partially invaginated into the LD, that link the LD to the tubular  
590 ER elements and that are often found close to mitochondria (Fig 3C). Although their  
591 origin was unknown, similar electron-dense structures were recently observed at LD-  
592 mitochondria contact sites (Ma et al., 2021). We reveal that these structures  
593 correspond to MAM and are strongly decreased in ORP5-depleted cells (Fig 3D). The

594 decrease in the LD population originating from MAM is accompanied by an increase in  
595 the LD population still linked to the ER but not in direct contact with mitochondria.

596 To conclude, our study uncovers an unprecedented role of ORP5 and ORP8 in  
597 orchestrating LD biogenesis at MAM. Our findings offer exciting perspectives in a more  
598 profound understanding of LD formation in cells and lipodystrophies, and neuronal  
599 disorders. Indeed, ORP5/8, seipin, and MAMs, which we now establish to localize in  
600 the same subdomains, are critical players of cellular lipid and calcium homeostasis,  
601 dysregulated in the onset of the above disorders.

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621 **MATERIAL AND METHODS**

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624 **Cell culture and transfection**

625 HeLa cells were cultured in DMEM (Life Technologies) containing GlutaMax (Life  
626 Technologies) and supplemented with 10% FBS (Life Technologies), 1%  
627 penicillin/streptomycin (Life Technologies), and 1% non-essential amino acids (Life  
628 Technologies) at 37°C and 5% CO<sub>2</sub>. For LD biogenesis experiments, HeLa cells were  
629 cultured in DMEM (Life Technologies) containing GlutaMax (Life Technologies) and  
630 supplemented with 5% lipoprotein-deficient serum FCS (Life Technologies), and 1%  
631 non-essential amino acids (Life Technologies) at 37°C and 5% CO<sub>2</sub> for 72 h, and then  
632 treated with BODIPY<sup>TM</sup> 558/568 (FA<sup>568</sup>) or oleic acid in serum depleted DMEM. For  
633 imaging, HeLa cells were seeded in 13-mm glass coverslips. HeLa cells were  
634 transfected with the indicated plasmids for 3h in serum depleted medium (Opti-MEM<sup>TM</sup>,  
635 ThermoFisher) using lipofectamine 2000 (Life Technologies), according to  
636 manufacturer's protocol. Cells were imaged 16-24hr hours post transfection.

637 Human hepatocarcinoma cells Huh7, were maintained in High Glucose with  
638 stabilized Glutamine and with Sodium Pyruvate Dulbecco's modified Eagle's Medium  
639 (DMEM) (Dutscher) supplemented with 10% heat-inactivated fetal bovine serum and  
640 1% penicillin/streptomycin (GibcoBRL). The Huh7 cells were transfected with indicated  
641 plasmid using Polyethyleneimine HCl MAX (Polysciences) following the  
642 manufacturer's instructions. For the swelling experiments, the Huh7 cells were first  
643 transfected with the plasmids and loaded with oleic acid for 24hr. Then, the culture  
644 media was next replaced by a hypotonic DMEM culture media, diluted twenty times by  
645 water. Cells were then imaged five minutes after the hypotonic medium addition.

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647

648 **siRNAs oligonucleotides**

649 Transient ORP5, ORP8 and PTPIP51 knockdowns in HeLa cells were performed  
650 by transfection of siRNA oligos using oligofectamine (Life Technologies) for 5 hours in  
651 serum depleted medium (Opti-MEM™, ThermoFisher), according to manufacturer's  
652 protocol. Cells were imaged 48 hours post transfection.

653 Double-stranded siRNAs were derived from the following references:

siRNA	Company, Reference
OSBPL8	Dharmacon, J-009508-06 (Galmes et al., 2016)
OSBPL8	Dharmacon, J-009508-05 (Galmes et al., 2016)
OSBPL5	Dharmacon, J-009274-10 (Galmes et al., 2016)
OSBPL5	Dharmacon, J-009274-11 (Galmes et al., 2016)
PTPIP51	Dharmacon, J-020973-10-0020 (Stoica et al., 2014)
Non-targeting	Dharmacon, D-001810-10

654

655 **Plasmids and cDNA clones**

656 EGFP-ORP5A, EGFP-ORP8, EGFP-ORP5 $\Delta$ PH, EGFP-ORP5 $\Delta$ TM, EGFP-ORD5  
657 and EGFP-ORD8 were described in (Galmes et al., 2016). mCh-Plin1 was described  
658 in (Ajjaji et al., 2019) and YFP-Seipin was described in (Santinho et al., 2020). GFP-  
659 Sec22b and RFP-Sec22b were described in (Gallo et al., 2020). GFP-Sec61 $\beta$  and  
660 ssHRP-KDEL were kindly gifted by T. Rapoport (Harward University) and D. Cutler  
661 (Schikorski et al., 2007), respectively. Mito-BFP (Addgene plasmid #49151;  
662 <http://n2t.net/addgene:49151>), Seipin-EGFP (Addgene plasmid #129719;  
663 <http://n2t.net/addgene:129719>) and TOM20-mCh (Addgene plasmid #55146;  
664 <http://n2t.net/addgene:55146>) were purchased from Addgene.

665

666 *Generation of the ORP5B variant by mutagenesis:*

667 The ORP5B natural variant (Du et al., 2020), partially deleted of its PH domain,  
668 was generated by site-directed mutagenesis (Quickchange II-XL, Stratagene). The  
669 oligos used were:

670 EGFP-ORP5B $\Delta$ 134-201\_Fw: CCTTTGGGGCCCTTCAGGCTGTCAGCCA

671 EGFP-ORP5B $\Delta$ 134-201\_Rv: TGGCTGACAGCCTGAAGGGCCCCAAAGG

672

673 *Generation of the ORP5/8 deletion mutants by mutagenesis:*

674 The CC and ORD domains of ORP5 or the TM domain of ORP8 were deleted  
675 using site-directed mutagenesis (Quickchange II-XL, Stratagene) to generate EGFP-  
676 ORP5 $\Delta$ CC and EGFP-ORP5 $\Delta$ ORD or EGFP-ORP8 $\Delta$ TM.

677 The following primers were used:

678 EGFP-ORP5 $\Delta$ ORD\_Fw:

679 CAGAGGAGAACAAGAGTCTGGAGGACCACAGCCCCTGGGAC

680 EGFP-ORP5 $\Delta$ ORD\_Rv:

681 GTCCCAGGGGCTGTGGTCCTCCAGACTCTTGTTCTCCTCTG

682 EGFP-ORP5 $\Delta$ CC 93-123\_Fw: CCCACCGCCAGGCCAGCGTGGTC

683 EGFP-ORP5 $\Delta$ CC 93-123\_Rv: GACCACGCTGGGCCTGGCGGTGGG

684 EGFP-ORP8 $\Delta$ TM\_Fw:

685 TATTTTCTGCAACAAAAAGACTAGGGGCCCGGGATC

686 EGFP-ORP8 $\Delta$ TM\_Rv:

687 GATCCCGGGCCCCTAGTCTTTTTGTTGCAGAAAATA

688 The EGFP-ORP5 $\Delta$ CC $\Delta$ TM and the EGFP-ORP5 $\Delta$ PH $\Delta$ TM were generated by  
689 deletion of the CC and of the PH in the EGFP-ORP5 $\Delta$ TM using site-directed  
690 mutagenesis (Quickchange II-XL, Stratagene). The oligos used to delete the CC are

691 listed above and the oligos used to delete the PH domain were described in (Galmes  
692 et al., 2016).

693

694 *Cloning of the RNAi-resistant ORP5 variants:*

695 RNAi-resistant EGFP-ORP5A and EGFP-ORP5B were generated by introducing 4  
696 silent point mutations in the region targeted by the 2 siRNA oligos (#10 and #11) by  
697 site-directed mutagenesis (Quickchange II-XL, Stratagene) and the following primers:

698 RESCUE ORP5\_siRNA10\_Fw:

699 GGG AAGGTCACCATCGAATGCGCGAAGAACA ACTTCCAGGCC

700 RESCUE ORP5\_siRNA10\_Rv:

701 GGCCTGGAAGTTGTTCTTCGCGCAITCGATGGTGACCTTCCC

702 RESCUE ORP5\_siRNA11\_Fw:

703 GAAGCCCAAGGGAATCAAGAAACCCTACAACCCCATCCTGGGGG

704 RESCUE ORP5\_siRNA11\_Rv:

705 CCCCAGGATGGGGTTGTAGGGTTICTTGATTCCCTTGGGCTTC

706 The untagged ORP5 $\Delta$ PH was generated from the RNAi-resistant EGFP-  
707 ORP5 $\Delta$ PH by excision of the EGFP using the enzymes NheI and HindIII followed by  
708 Klenow polymerase treatment and ligation using a T4 DNA ligase.

709

710 *Cloning of HA-ORP5wt and HA-ORP5 $\Delta$ PH:*

711 To generate the HA-ORP5wt, the PCR product, carrying the HA tag at the N-  
712 terminus of ORP5, was ligated between AgeI and XhoI in the pEGFP-C1 vector  
713 (Clontech) to replace the GFP- with the HA-tag. The oligos used are:

714 ORP5 HA Age1\_Fw:

715 5'GGCGGCACCGGTcgccaccATGTACCCATACGATGTTCCAGATTACGCTatgaagg  
716 aggaggccttcctc 3'

717 ORP5 Xho1\_Rv: 5' GGCCTCGAGctatttgaggatgtgggtaatg 3'

718 To generate the HA-ORP5  $\Delta$ PH, the PH domain in HA-ORP5wt was deleted by site-  
719 directed mutagenesis (Quickchange II-XL, Stratagene) as in (Galmes et al., 2016).

720

#### 721 *Cloning of Opi1 pQ2S-PABd*

722 Opi1 pQ2S-PABd probe was generated by inserting the fragment 113-168 from  
723 Opi1p (GeneBank M57383.1) using Yeast DNA (gift from Dr. S. Friant, Université de  
724 Strasbourg, France) in *Bgl*II and *Eco*RI digested pEGFP-C1. Specificity for binding PA  
725 was established using a liposome binding assay as described in (Kassas et al., 2017).

726

#### 727 *Cloning of RFP-ORP5 $\Delta$ TM and RFP-ORP5B*

728 The insert RFP was recovered from a plasmid mTAG-RFP digested with the  
729 enzymes *Nhe*I (Fast Digest Thermo)/*Bs*GrI(Fast Digest Thermo) or *Nhe*I/*Xho*I (Fast  
730 Digest Thermo). Meanwhile, the plasmids EGFP-ORP $\Delta$ TM and EGFP-ORP5B were  
731 respectively digested with the enzymes *Nhe*I/*Bsr*GI and *Nhe*I/*Xho*I to remove the tag  
732 EGFP. The insert RFP was then ligated on the plasmid without the tag.

733

#### 734 **Antibodies, probes and reagents**

735 Primary antibodies used in this study were following: rabbit anti-ORP5 (SIGMA,  
736 HPA038712), mouse anti-ORP8 (Santa Cruz, sc-134409), rabbit anti-PTPIP51  
737 (RDM3, SIGMA, HPA009975), mouse anti-PTPIP51 (FAM82C, SIGMA,  
738 SAB1407626), mouse anti-GAPDH (Genetex, GTX627408), rabbit anti-GFP



739 (Invitrogen, A11122) and mouse anti-HA (SIGMA, H3663). Dilutions are detailed in the  
740 table below.

741 Mitotracker red (ThermoFisher) and LipidTox<sup>TM</sup> (LTox, ThermoFisher) were used  
742 as probes for the mitochondrial network and the LDs, respectively, following the  
743 manufacturer's instructions. Additionally, in LD biogenesis experiments, LDs were  
744 labeled by BODIPY<sup>TM</sup> 558/568 (FA<sup>568</sup>, fluorescent tagged oleic acid, Thermofisher) or  
745 LD540 (Spandl et al., 2009). Oleic acid-Albumin from bovine serum (OA, SIGMA,  
746 03008) was used to induce LD production.

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Rabbit anti-ORP5	IF 1:300 PLA 1:200
Mouse anti-ORP8	PLA 1:200
Rabbit anti-PTPIP51	PLA 1:200 WB 1:800
Mouse anti-PTPIP51	PLA 1:200
Mouse Anti-GAPDH	WB 1:10000
Rabbit anti-GFP	IEM 1:100
Mouse anti-HA	IEM 1:500
Mitotracker	1 $\mu$ M
LipidTOX <sup>TM</sup> (LTox)	10 $\mu$ M
BODIPY <sup>TM</sup> 558/568 (FA <sup>568</sup> )	1 $\mu$ M
Oleic Acid (OA)	300 $\mu$ M
LD450	10 $\mu$ M

758

### 759 **LD Biogenesis and oleic acid treatment**

760 LD biogenesis was induced in delipidated HeLa cells grown on coverslips by  
761 treatment with FA<sup>568</sup> or OA in serum depleted DMEM. For LD biogenesis time-course  
762 experiments and LD biogenesis recue experiments, delipidated HeLa cells (control and  
763 knockdown for ORP5, ORP8 or PTPIP51) expressing Mito-BFP alone or Mito-BFP

764 together with EGFP tagged-ORP5 were treated with 1  $\mu$ M FA<sup>568</sup> and then fixed in  
765 4%PFA at different times. After fixation, LDs were stained with LTox for 30 min in PBS  
766 and mounted for observation. A subset of LD biogenesis time-course experiments  
767 were performed in delipidated HeLa cells (control and knockdown for ORP5 or ORP8)  
768 treated with 300  $\mu$ M OA and in which mitochondrial network was labelled with  
769 Mitotracker. For all other experiments, non-delipidated HeLa cells were treated with  
770 300  $\mu$ M OA for 2hr before fixation.

771

### 772 **Confocal Microscopy**

773 Images of immunostained cells or cells expressing fluorescent tagged proteins  
774 were acquired on Confocal inverted microscope SP8-X (DMI 6000 Leica). Optical  
775 sections were acquired with a Plan Apo 63x oil immersion objective (N.A. 1.4, Leica)  
776 using the LAS-X software. Fluorescence was excited using either a 405nm laser diode  
777 or a white light laser, and later collected after adjusting the spectral windows with  
778 GaAsP PMTs or Hybrid detectors. Images from a mid-focal plane are shown. For Huh7  
779 experiments, images were acquired by the ZEISS LSM800 Airyscan.

780

### 781 **Live-cell Imaging**

782 HeLa cells were seeded on glass bottom ibidi chambers ( $\mu$ -slide 2 wells) 2 days  
783 before imaging. The day after seeding, EGFP-ORP5B and Mito-BFP plasmids were  
784 transfected with of lipofectamine 2000/well (Invitrogen), according to the  
785 manufacturer's instructions. Cell imaging was performed on an inverted Nikon Ti  
786 Eclipse E microscope coupled with a Spinning Disk (Yokogawa CSU-X1-A1) and cage  
787 incubator to control both temperature and CO<sub>2</sub> (37 °C, 5% CO<sub>2</sub>). After excitation with  
788 a 405nm (Vortran, 100 mW), 491nm (Vortran, 150 mW) and 561 nm laser (Coherent,

789 100 mW), fluorescence from the different fluorescent compounds was detected with a  
790 40X oil immersion objective (PLAN FLUOR; NA: 1.30; Nikon), an emission filter Quad  
791 bandpass 440/40 nm, 521/20 nm, 607/34 nm, 700/45 nm (Semrock) and a Prime 95B  
792 sCMOS camera (Photometrics). Images were captured every 30 sec during 15 min.  
793 Approximately 2 min after the start of captures, FA<sup>568</sup> was added to a final  
794 concentration of 1  $\mu$ M to induce the formation of lipid droplets. For live imaging, Huh7  
795 cells were grown in MatTek 3.5mm coverslip bottom dishes and imaged on the ZEISS  
796 LSM800 Airyscan microscope.

797

#### 798 **Quantifications:**

799 *Colocalization analysis:* for co-localization analysis of fluorescent signals, the  
800 acquired images were processed using the JACoP plugin in ImageJ to assess the  
801 Pearson's correlation coefficient. The obtained values, ranging from 0 to 1 (1=max  
802 correlation), indicated the association between the signals analysed.

803 *Organelles/structures count:* LTox- and FA-positive LDs and ORP5 labeled-ER  
804 were counted in maximal projection confocal images using ImageJ software.

805 *Nearest-neighbor distance analysis:* the probability densities of distance  
806 distribution in Figure S5D was performed by the Mosaic Interaction analysis plug-in for  
807 Fiji (Shivanandan et al., 2013).

808 *Imaris Analysis: quantification of PLA and seipin spots:* in order to quantify PLA  
809 spots and categorize seipin spots in HeLa cells, confocal images were treated with the  
810 surface function of the software IMARIS (Bitplane, v9.3.1). For PLA quantification, 3D  
811 images (PLA foci identified as "spots", mitochondria identified as "surfaces) were  
812 generated from confocal Z-stack images and the shortest distance between each spot  
813 center and the nearest point of the surface or cell object was calculated based on a 3D

814 distance map. Spots objects (PLA dots) with a distance smaller than 380nm from  
815 surfaces (mitochondria) objects were considered at a close proximity of these objects.  
816 The threshold of 380 nm was used as an estimation of the PLA reaction precision  
817 including both primary and secondary antibodies (30nm) plus half the FWHM of the  
818 PLA amplification signals (350nm). Similarly, 3D segmented images of HeLa cells co-  
819 expressing seipin and Mito-BFP, and stained with LTox were generated from z-stack  
820 confocal images (seipin, mitochondria and LD identified as “surfaces). Seipin 3D  
821 surfaces were then classified into 4 different categories (“seipin to MAM”, “seipin to  
822 LD”, “seipin to MAM-LD” and “seipin to ER”) according to their proximity to the labeled  
823 compartments using a 3D distance map. The results as showed as the percentage of  
824 the category to the total number of the green surfaces.

825

### 826 **3D Structured Illumination Microscopy (SIM)**

827 Super-resolution light microscopy was performed on a Zeiss ELYRA PS.1 SIM  
828 microscope, equipped with a Plan-Apochromat 63x/1.40 NA oil-immersion objective  
829 (Carl Zeiss). The illumination patterns of the 488, 561 and 642 nm lasers were  
830 projected into the sample. The emitted fluorescence light was detected with an  
831 EMCCD camera (iXon 885, Andor Technology). Five phase translations and three  
832 rotations of the illumination pattern were recorded at each z-plan and image stacks  
833 (120-nm increment along z axis) were acquired. The 3D stacks were then  
834 computationally reconstructed with the ZEN imaging software package (algorithm of  
835 Heintzmann and Cremer) to generate super-resolution 3D SIM images with twofold  
836 extended resolution in the three axes (reconstructed image format = 1904 x 1900  
837 pixels, representing voxels of 0.04 x 0.04 x 0.12  $\mu\text{m}$ ).

838

839           **Immunofluorescence**

840           Labeling of mitochondrial network with Mitotracker was performed by incubating  
841 HeLa cells seeded in glass coverslips with a Mitotracker red 1  $\mu$ M in Opti-MEM™  
842 (ThermoFisher) for 30 min at 37°C and 5% CO<sub>2</sub>. Cells fixation was carried out by  
843 incubation with 4% PFA/PBS for 30 min at room temperature. Coverslips were then  
844 washed in PBS and incubated with 50 mM NH<sub>4</sub>Cl/PBS for 15 min at room temperature.  
845 For immunofluorescence, cells were incubated with the primary antibodies diluted in  
846 blocking solution (1% BSA / 0.1% Saponin in PBS) for 1 hr at room temperature.  
847 Coverslips were thereafter incubated with fluorescently-labeled secondary antibodies  
848 (1:500 in BB) for 1hr at room temperature. To label LDs, cells were incubated with 10  
849  $\mu$ M LTox in 1xPBS for 30 minutes at room temperature. After washing with PBS,  
850 coverslips were mounted with Vectashield (Vectro Laboratories) on microscopy slides.  
851 Images were acquired on a confocal inverted microscope SP8-X (DMI 6000 Leica).  
852 Optical sections were acquired with a Plan Apo 63x oil immersion objective (N.A. 1.4,  
853 Leica) using the LAS-X software. Fluorescence was excited using either a 405nm laser  
854 diode or a white light laser, and later collected after adjusting the spectral windows with  
855 GaAsP PMTs or Hybrid detectors. Images from a mid-focal plane are shown.

856

857           ***In situ* proximity ligation assay (PLA)**

858           The protein-protein interactions in fixed HeLa cells were assessed using *in situ*  
859 PLA (Duolink®SIGMA) according with the manufacturer's instructions. After fixation,  
860 HeLa cells co-expressing Mito-BFP and mCh-Plin1, were incubated with primary  
861 antibodies, mouse anti-ORP8 (1:200) plus rabbit anti-ORP5 (1:200), mouse anti-ORP8  
862 (1:200) plus rabbit anti-PTPIP51 (1:200), or rabbit anti-ORP5 (1:200) plus mouse anti-  
863 PTPIP51 (1:200), in blocking solution (1% BSA, w/v 0.01% saponin, w/v, in PBS) for

864 1hr at room temperature. PLUS and MINUS PLA probes (anti-murine and anti-rabbit  
865 IgG antibodies conjugated with oligonucleotides, 1:5 in blocking solution) were then  
866 incubated with the samples for 1hr at 37°C. Coverslips were thereafter washed in 1x  
867 wash buffer A and incubated with ligation solution (5x Duolink<sup>®</sup> Ligation buffer 1:5,  
868 ligase 1:40 in high purity water) for 30 minutes at 37°C. After the ligation step, cell  
869 samples were washed in 1x wash buffer A and incubated with the polymerase solution  
870 (5x Amplification buffer 1:5, polymerase 1:80 in high purity water) for 1h40min at 37°C.  
871 Polymerase solution was washed out from the coverslips with 1x wash buffer B and  
872 0.01x wash buffer B. Vectashield Mounting Medium (Vector Laboratories) was used  
873 for mounting.

874

## 875 **Electron Microscopy Analysis**

### 876 *Conventional EM:*

877 For conventional EM, cells grown on 13 mm glass bottom coverslips (Agar  
878 Scientific) were fixed with 2.5% glutaraldehyde and 2% PFA in 0.1 M cacodylate,  
879 0.05% CaCl<sub>2</sub> buffer for 24 hours. After several washes with 0.1 M cacodylate buffer,  
880 the cells were postfixated with 1% OsO<sub>4</sub>, 1.5% potassium ferricyanide in 0.1M  
881 Cacodylate for 1 hour. After several washes with 0.1 M cacodylate buffer and H<sub>2</sub>O, the  
882 cells were stained with 0.5% uranyl acetate for 24 hours. After several washes with  
883 H<sub>2</sub>O, the cells were dehydrated in ethanol and embedded in Epon while on the  
884 coverslips. Ultrathin sections were prepared, counterstained with uranyl acetate and  
885 observed under a 80kV JEOL 1400 microscope equipped with a Orius High speed  
886 (Gatan) camera.

### 887 *HRP Detection:*

888 HeLa cells expressing HRP-KDEL were fixed on coverslips with 1.3%  
889 glutaraldehyde in 0.1 M cacodylate buffer, washed in 0.1 M ammonium phosphate [pH  
890 7.4] buffer for 1 hour and HRP was visualized with 0.5 mg/ml DAB and 0.005% H<sub>2</sub>O<sub>2</sub>  
891 in 0.1 M Ammonium Phosphate [pH 7.4] buffer. Development of HRP (DAB dark  
892 reaction product) took between 5 min to 20 min and was stopped by extensive washes  
893 with cold water. Cells were postfixed in 2% OsO<sub>4</sub>+1% K<sub>3</sub>Fe(CN)<sub>6</sub> in 0.1 M cacodylate  
894 buffer at 4°C for 1 hour, washed in cold water and then contrasted in 0.5% uranyl  
895 acetate for 2 hours at 4°C, dehydrated in an ethanol series and embedded in epon as  
896 for conventional EM. Ultrathin sections were contrasted with 4% uranyl acetate and  
897 observed under a FEI Tecnai 12 microscope equipped with a OneView 4k Gatan  
898 camera.

899 *Serial sectioning and 3D reconstruction:*

900 Ultrathin serial sections (50nm) of HRP-KDEL-transfected HeLa cells were cut and  
901 put on slot grids (EMS) with formvar (1%) and then contrasted with 4% uranyl acetate.  
902 13 and 14 serial sections for ORP5wt and ORP5ΔPH, respectively, were collected and  
903 observed under a 80kV JEOL 1400 microscope equipped with a Orius High speed  
904 (Gatan) camera. Image alignment was done with imod and segmentation was done  
905 manually by using 3Dmod.

906 *Immunogold labelling and quantifications:*

907 HeLa cells were fixed with a mixture of 2%PFA and 0.125% glutaraldehyde in  
908 0.1 M phosphate buffer [pH 7.4] for 2 hours, and processed for ultracryomicrotomy as  
909 described previously (Slot and Geuze, 2007). Ultrathin cryosections were single- or  
910 double-immunogold-labeled with antibodies and protein A coupled to 10 or 15 nm gold  
911 (CMC, UMC Utrecht, The Netherlands), as indicated in the legends to the figures.  
912 Immunogold-labeled cryosections were observed under a FEI Tecnai 12 microscope

913 equipped with a OneView 4k Gatan camera. For the quantification of the distribution  
914 of seipin immunogold labeling on ultrathin cryosections, gold particles (15nm) were  
915 counted on acquired micrographs of randomly selected cell profiles (the number of cell  
916 profiles and of gold particle is indicated in the figure legends). All data are presented  
917 as mean (%)  $\pm$ SEM of three technical replicates.

918

### 919 **Interfacial tension measurements**

920 Interfacial tension measurements were performed using a drop tensiometer device  
921 designed by Teclis Instruments (Tracker, Teclis-IT Concept, France) to measure the  
922 interfacial tension of oil – water interfaces. In our experiments, the pendant drop is the  
923 triolein lipid phase, formed in the aqueous HKM buffer. The triolein – water interface  
924 stabilizes at  $\sim 34 \pm 1$  mN/m. Adsorption of ORD5/8 translated into a decrease in tension,  
925 as the protein got recruited to the oil-water interface. Throughout the adsorption  
926 kinetics to either a triolein – water interface, the drop area was constant.

927

### 928 **PIP-Strip**

929 PIP Strips membranes (Echelon Biosciences) were blocked with 3% BSA FFA  
930 dissolved in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (3% BSA  
931 FFA PBS-T) at room temperature for 60 min.

932 Blocked PIP Strips were incubated with the same buffer containing the <sup>ORP5</sup>HA-  
933 CC, <sup>ORP8</sup>HA-CC or HA peptide (negative control) at final concentration 0.35  $\mu$ M for 1h.  
934 The PIP strips were then washed and bounded proteins were detected with Rabbit anti  
935 HA antibody.

936

937



938 **Statistical Analysis**

939 The Kolmogorov-Smirnov test was used for the tensiometer studies. The  
940 Wilcoxon-Mann-Whitney test was used for the quantification analysis of seipin by  
941 confocal imaging. The unpaired two-tailed *t*-test for all the other experiments.

942

943 **AUTHOR CONTRIBUTIONS**

944 FG and ART designed the work. VG, VC and MO performed and analyzed most  
945 of the confocal imaging experiments. VG and MO performed the live cell imaging  
946 experiments. VC performed and analyzed the LD biogenesis experiments and the PLA.  
947 VG performed the seipin localization analysis by Imaris. MO performed the imaging on  
948 Huh7 and the PIP strip experiments. CS performed and analyzed some of the LD  
949 biogenesis experiments. AH, VG and VC generated ORP5 mutants and performed  
950 confocal analysis of their localization. AH and FG performed and analyzed the electron  
951 microscopy experiments. CB performed the serial sectioning-3D EM. OF performed  
952 the structured illuminated microscopy. NV generated and provided tools for PA imaging  
953 analysis. KBM performed the interfacial tension measurements. NEK assisted in  
954 western blot analysis, electron microscopy and generated some of the constructs for  
955 mammalian cell expression. FG and ART wrote the manuscript and all authors  
956 contributed to improve the manuscript.

957

958 **CONFLICT OF INTERESTS**

959 The authors declare that they have no competing interests.

960

961 **DATA AVAILABILITY SECTION**

962 This study includes no data deposited in external repositories.

963

964

965           **REFERENCES**

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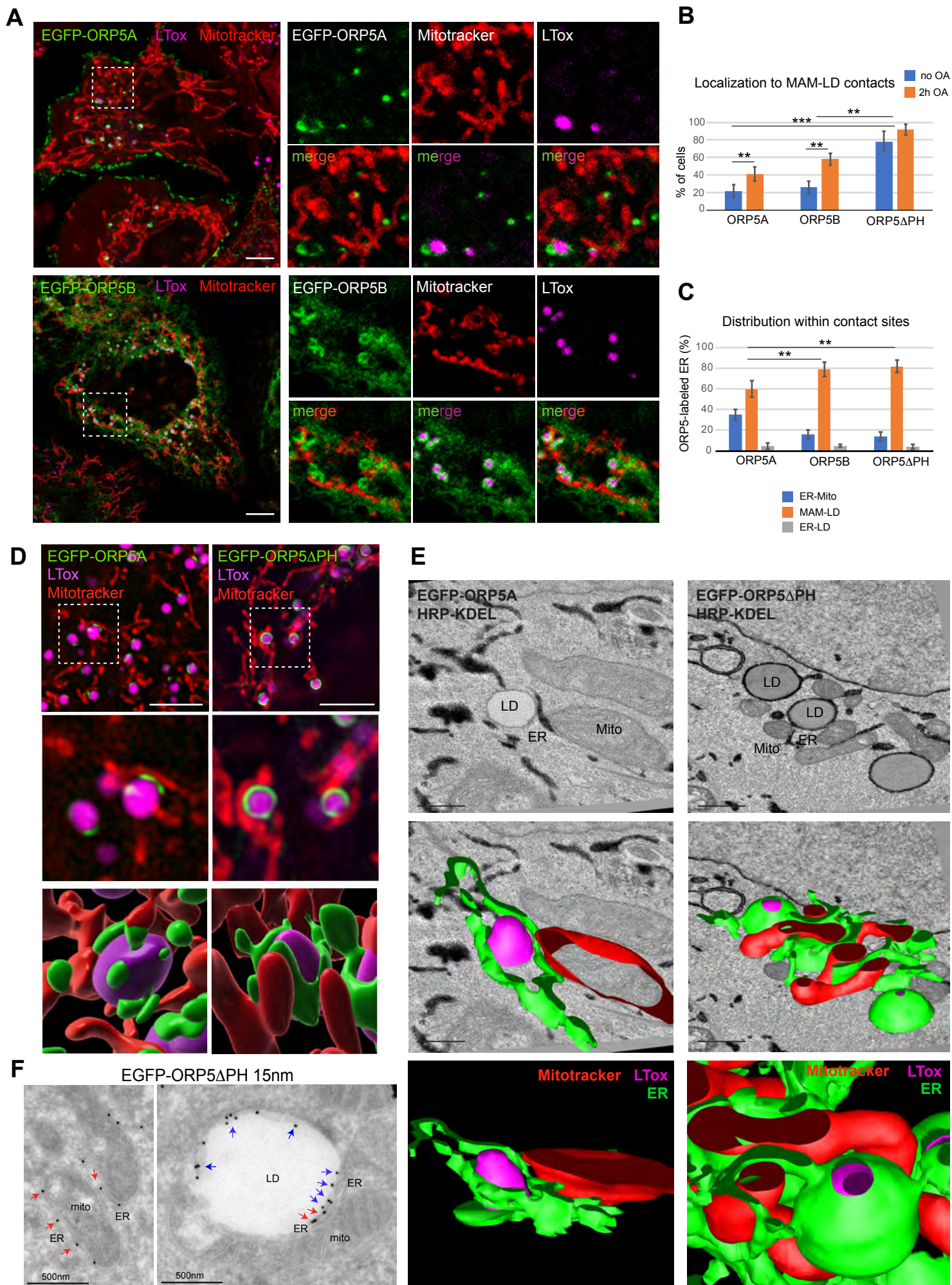
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## FIGURES AND FIGURE LEGENDS



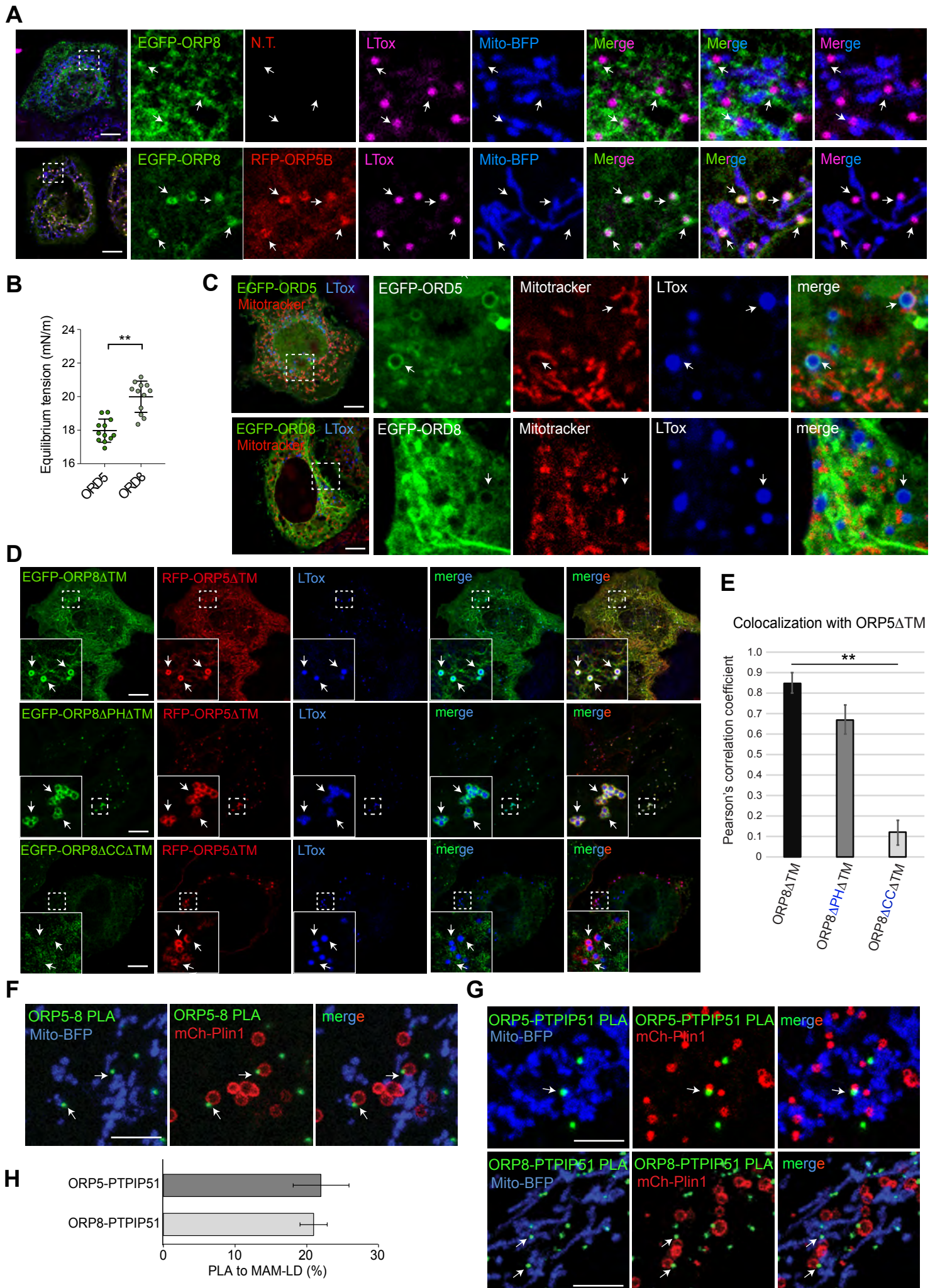


**Figure 1**

**Figure 1. ORP5 localizes to MAM subdomains in contact with LD**

**A)** Representative confocal images showing single focal planes of HeLa cells expressing EGFP-ORP5A or EGFP-ORP5B (green) and treated with Mitotracker (red) and LTox Deep Red (LTox, purple) to label mitochondria and lipid droplets (LD), respectively. Scale bar 10  $\mu\text{m}$ . **B)** Quantification of the % of HeLa cells showing localization of EGFP-tagged ORP5A, ORP5B and ORP5 $\Delta$ PH to MAM-LD contacts, in absence of Oleic acid (OA) treatment or after 2hr of 300  $\mu\text{M}$  OA treatment. Data represent the mean $\pm$ standard error of the mean (SEM) of n= 25 cells. \*\* p<0.01, \*\*\*p<0.001, unpaired two-tailed *t*-test. **C)** Analysis of the distribution of EGFP-tagged ORP5A, ORP5B and ORP5 $\Delta$ PH among the contact sites ER (MAM)-mitochondria (Mito), MAM-LD and ER-LD in HeLa cells treated with 300  $\mu\text{M}$  OA for 2hr. Data represent the mean $\pm$ SEM of n=15 cells. \*\* p<0.01. **D)** Structured illumination microscopy (SIM) micrographs of HeLa cells expressing EGFP-ORP5B or EGFP-ORP5 $\Delta$ PH (green), treated with OA for 2hr, and stained with Mitotracker (red) and LTox Deep Red (purple). 3D-SIM images were obtained by segmentation using software Imaris (v 9.3, Bitplane). Scale bar, 5 $\mu\text{m}$ . **E)** Representative electron micrographs of HeLa cells co-overexpressing EGFP-ORP5A or EGFP-ORP5 $\Delta$ PH and HRP-KDEL after 2hr OA treatment, and their 3D representation obtained by 3Dmod. LD, lipid droplet, Mito, mitochondria, and ER, endoplasmic reticulum. Scale bar 500nm. **F)** Electron micrograph of ultrathin cryosections of HeLa cells transfected with EGFP-ORP5 $\Delta$ PH, treated with OA for 2hr, and immunogold stained with anti-EGFP (15 nm gold). Left: red arrows indicate EGFP-ORP5 $\Delta$ PH localized to MAM. Right: blue arrows indicate ORP5 $\Delta$ PH localized to ER-LD contacts and red arrows indicate ORP5 $\Delta$ PH localized to MAM-LD contacts. Scale bar 500 nm.



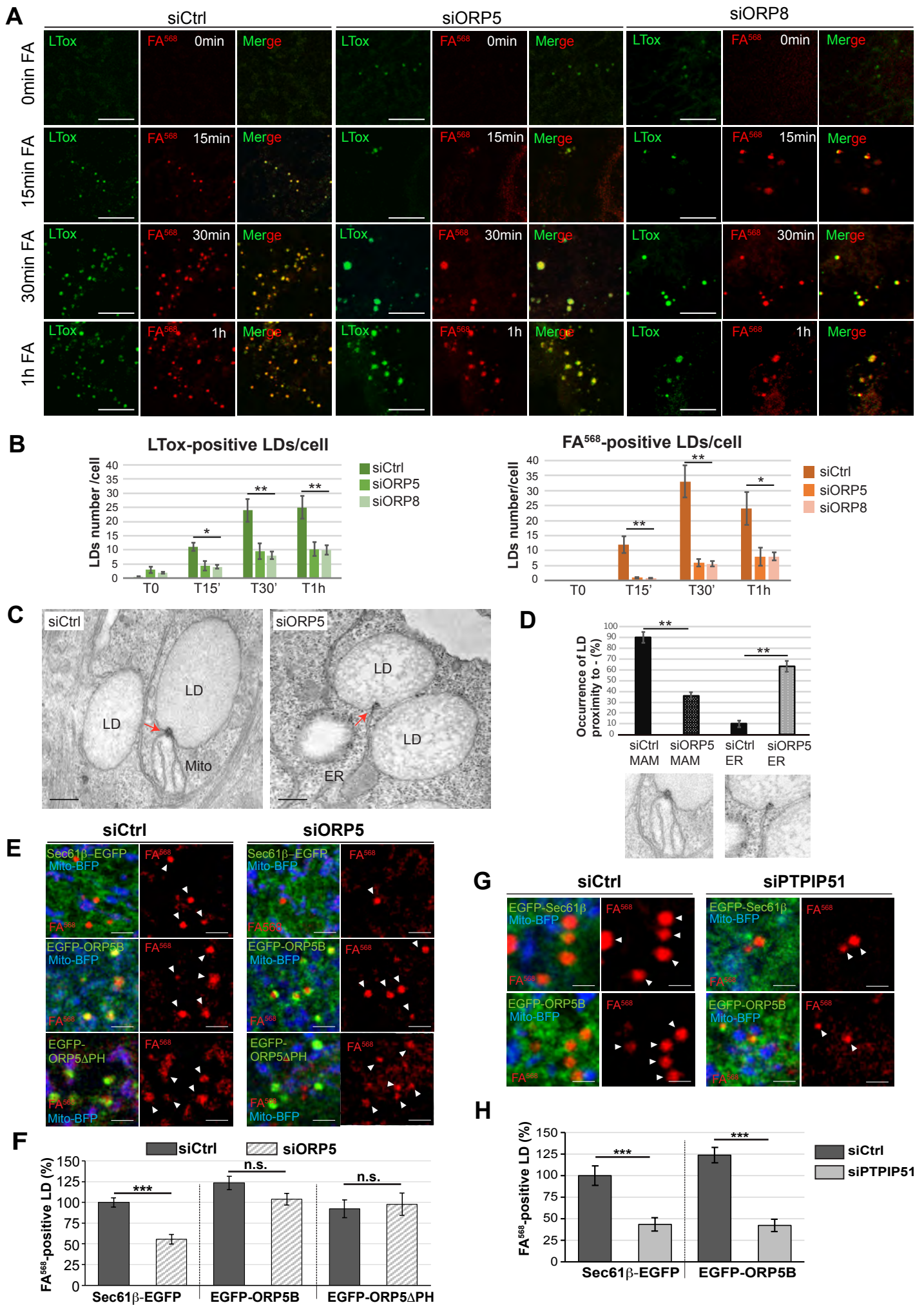


**Figure 2**

## Figure 2. ORP8 localizes and interacts with ORP5 at Mito-MAM-LD contacts

**A)** Representative confocal images showing localization of EGFP-ORP8 alone (green) or together with RFP-ORP5B (red), in HeLa cells co-expressing Mito-BFP (blue), and treated with OA for 2hr and stained with LTox Deep Red (purple). Each image represents a single focal plane of confocal 3D stacks. Arrows point to ORP5-labeled MAM associated to mitochondria and LD (MAM-LD contacts). Scale bar 10  $\mu$ m. **B)** LD-binding affinity of ORP5 and ORP8 ORD domains (ORD5 and ORD8, respectively) analyzed by tensiometry. Data are shown as mean $\pm$ SEM of n=12, with \*\* p<0.01, unpaired Kolmogorov-Smirnov test. **C)** Confocal images showing the localization of EGFP-ORD5 and EGFP-ORD8 (green) in HeLa cells treated with OA for 2hr and stained with Mitotracker (red) and LTox Deep Red (blue). Each image represents a single focal plane, scale bar 10  $\mu$ m. **D)** HeLa cells co-expressing EGFP-ORP8 $\Delta$ TM and RFP-ORP5 $\Delta$ TM, EGFP-ORP8 $\Delta$ PH $\Delta$ TM and RFP-ORP5 $\Delta$ TM, or EGFP-ORP8 $\Delta$ CC $\Delta$ TM and RFP-ORP5 $\Delta$ TM. Each image represents a single focal plane of confocal 3D stacks. Arrows point to ORP5 localization on the LD surface. Scale bar 10  $\mu$ m. **E)** Quantitative analysis of the co-localization of either EGFP-ORP8 $\Delta$ TM, EGFP-ORP8 $\Delta$ PH $\Delta$ TM or EGFP-ORP8 $\Delta$ CC $\Delta$ TM with RFP-ORP5 $\Delta$ TM by Pearson's correlation coefficient. Data represent mean $\pm$ SEM of n=10 cells. \*\*p<0.001 unpaired student's *t*-test. **F-G)** Confocal micrographs showing endogenous ORP5-ORP8, ORP5-PTPIP51 and ORP8-PTPIP51 PLA interactions (green dots) in regions of HeLa cells co-expressing Mito-BFP (blue) and mCherry-Plin1 (mCh-Plin1) and treated with 300  $\mu$ M OA for 2hr. Arrows point to PLA dots associated to MAM-LD contacts. Images represents a single focal plane. Scale bar 10  $\mu$ m. **H)** Quantification of endogenous ORP5-PTPIP51 and ORP8-PTPIP51 PLA interaction at MAM-LD contacts. Data is shown as % mean $\pm$ SEM of n= 36 cells (ORP5-PTPIP51) and n=15 cells (ORP8-PTPIP51).

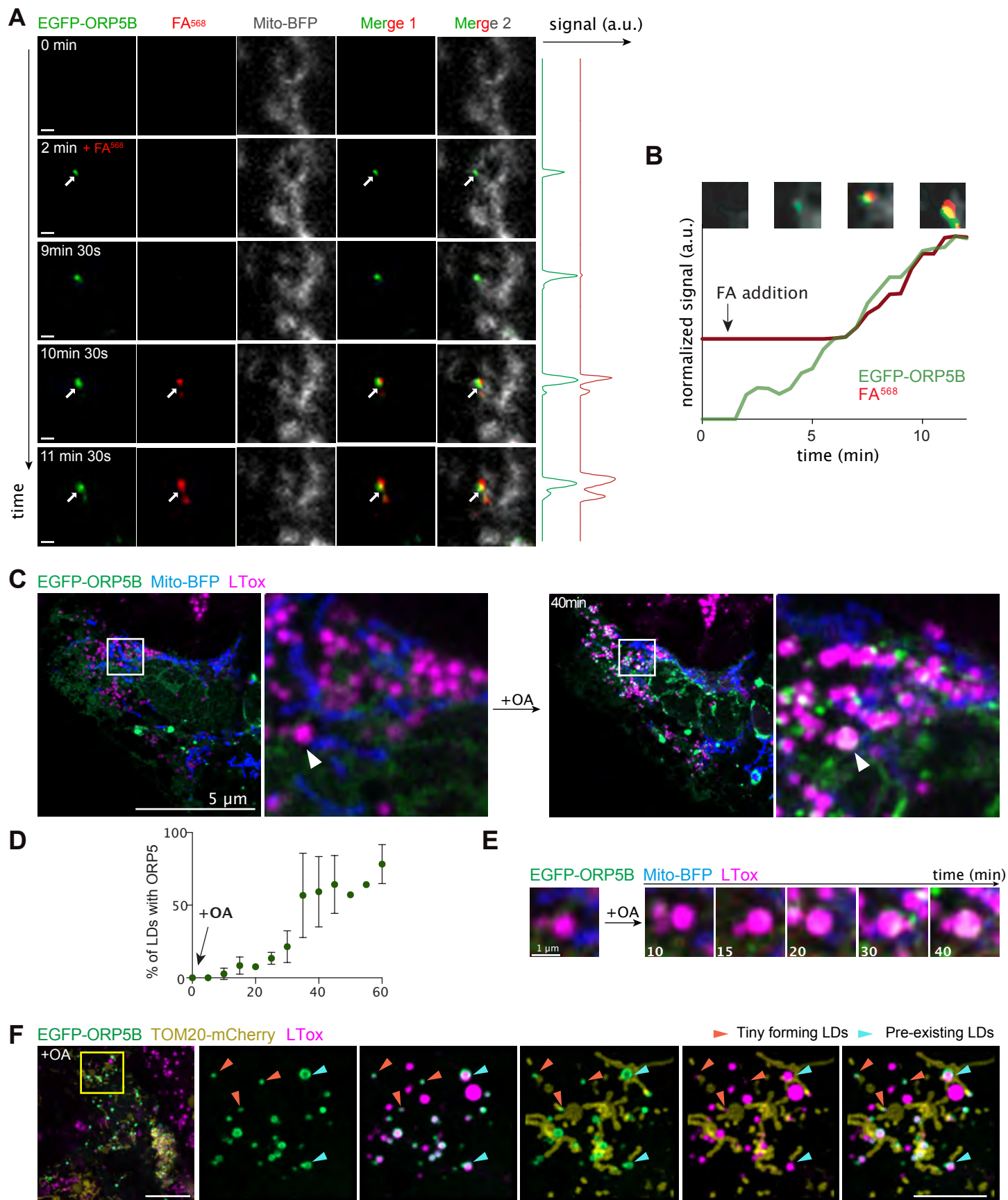




**Figure 3**

### Figure 3. Depletion of ORP5 and ORP8 affect LD biogenesis

**A)** LD biogenesis time-course. HeLa cells delipidated for 72hr were treated with siCtrl, siORP5 or siORP8, incubated with 1 $\mu$ M FA<sup>568</sup> (red) and stained with LTox Deep Red (green). Representative confocal images of regions of HeLa cells submitted to these experimental conditions at time 0 min, 15 min, 30 min and 1 hr of FA<sup>568</sup> incubation are displayed as single focal plane. Scale bar 5  $\mu$ m. **B)** Quantification of the number of FA<sup>568</sup>- and LTox-positive LD in control, ORP5 and ORP8 knockdown HeLa cells at the indicated times. Data represent mean $\pm$ SEM of n= 30 cells. \*p<0.001, \*\*p<0.0001, unpaired two-tailed *t*-test. **C)** Representative electron micrographs of control and ORP5 knockdown HeLa cells, evidencing (red arrows) the electrondense structure that connects the nascent LD to the ER from which it originated and sometimes also to the mitochondria (Mito) at MAM-LD contacts. Scale bar 250 nm. **D)** Quantification of the number of LD associated to these ER or MAM electrondense structures. Data represent mean $\pm$ SEM of n= 20 cells. \*\*p<0.0001, unpaired two-tailed *t*-test. **E)** Confocal (single focal plane) micrographs of regions of control and ORP5 knockdown delipidated HeLa cells co-overexpressing Mito-BFP (blue) with either Sec61 $\beta$ -EGFP (green), or EGFP-ORP5B (green), or EGFP-ORP5 $\Delta$ PH (green). Arrowheads indicate the newly formed LD. Scale bar, 2  $\mu$ m. **F)** Quantitative analysis of the number of FA<sup>568</sup>-positive LD in control and ORP5 knockdown delipidated HeLa cells co-overexpressing Mito-BFP and either Sec61 $\beta$ -EGFP, or siRNA-resistant EGFP-ORP5B or EGFP-ORP5 $\Delta$ PH, and treated for 15 min with FA<sup>568</sup>. Data are shown as % of mean $\pm$ SEM of n=20-85 cells. \*\*\*p<0.001, unpaired two-tailed *t*-test. **G)** Confocal (single focal plane) micrographs of regions of control and PTPIP51 knockdown delipidated HeLa cells, co-overexpressing Mito-BFP (blue) with Sec61 $\beta$ -EGFP (green) or EGFP-ORP5B (green) and treated for 1hr with FA<sup>568</sup>. Arrowheads indicate the newly formed LD. Scale bar, 1  $\mu$ m. **F)** Quantification of the number of FA<sup>568</sup>-positive LD in control and PTPIP51 knockdown delipidated HeLa cell co-overexpressing Mito-BFP and either Sec61 $\beta$ -EGFP or EGFP-ORP5B and treated for 1hr with FA<sup>568</sup>. Data are shown as % of mean $\pm$ SEM of n=20-22 cells. \*\*\*p<0.001, unpaired two-tailed *t*-test.

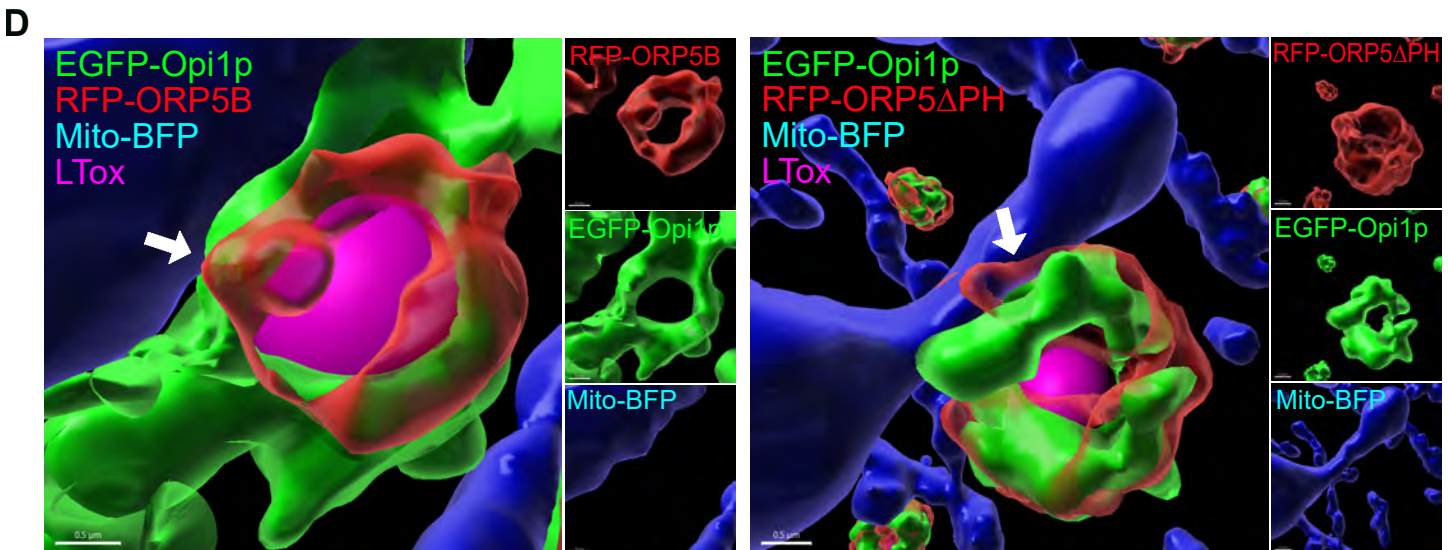
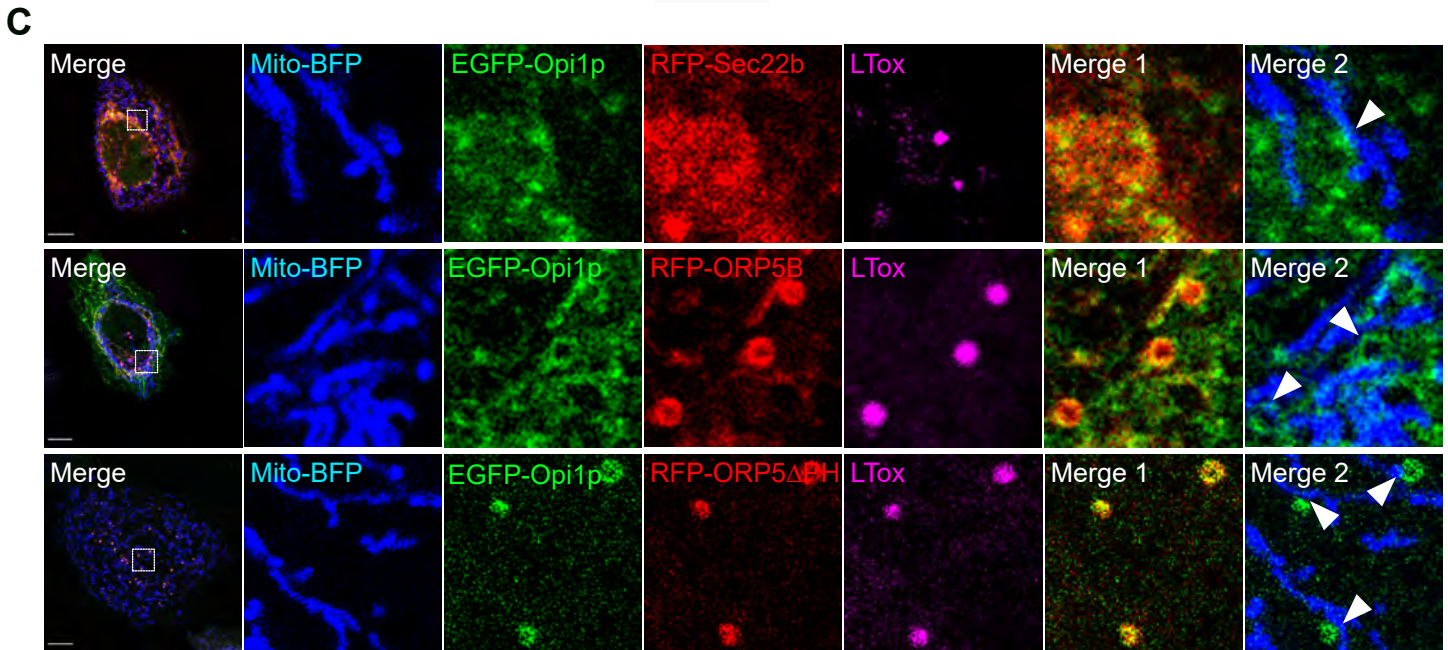
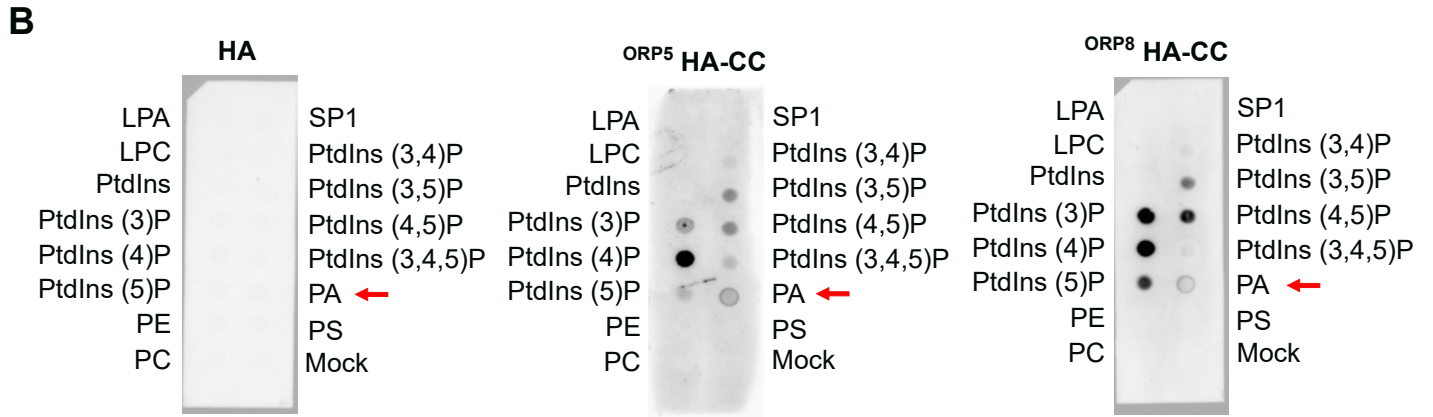
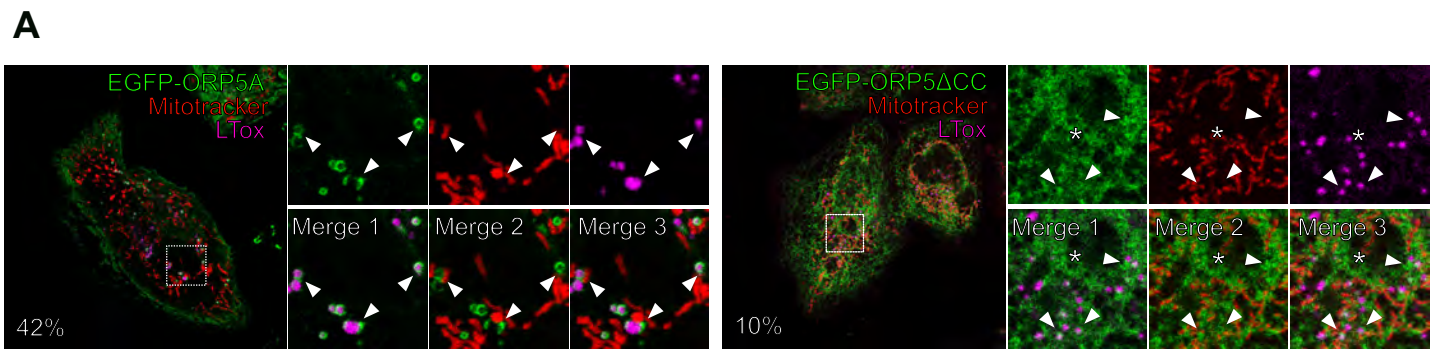


**Figure 4**

**Figure 4. ORP5 specifically localizes to ER subdomains where LDs originate and also to the preexisting lipid droplets**

**A)** Zoom of spinning video snapshots of HeLa cells expressing EGFP-ORP5B (green) and Mito-BFP (grey). After 2 min of acquisition, the cells were treated with FA<sup>568</sup> at 1 μM. Arrows indicate ORP5-labeled MAM-LD contacts associated to mitochondria. Full cell view in supplementary figure 5A. Scale bar, 1 μm. **B)** Full time course analysis of the intensity changes for ORP5B (green) and FA<sup>568</sup> (red) over time. **C)** Example of an Airyscan video snapshots of Huh7 cells expressing EGFP-ORP5B (green), RFP-Sec22b (red, shown in supplementary Figure 5B) and Mito-BFP (blue) before and after 40 min of 200 μM OA treatment. The lipid droplets were stained using LTox Deep Red (purple). Arrowheads indicate absence or presence of ORP5B at MAM-LD contacts before and after OA treatment, respectively. Full sequence in supplementary Figure 5B. Scale bar =10 μm (entire cell) or 5 μm (zoom) **D)** Quantification of the number (%) of LDs with EGFP-ORP5 over the indicated time points. **E)** Time course of ORP5 recruitment to a large pre-existing LDs depicted by the white arrowhead in the Huh7 cell in (C). Scale bar 1 μm. **F)** Representative Airyscan snapshot of Huh7 cells expressing EGFP-ORP5B (green) and TOM20-mCherry (yellow), staining mitochondria, after 1h30min of 200 μM OA treatment. The lipid droplets were stained using LTox Deep Red (purple). Scale bar, 10 μm (entire cell) or 5 μm (zoom). Orange arrowheads indicate tiny emerging LDs, light green arrowheads indicate pre-existing LDs.



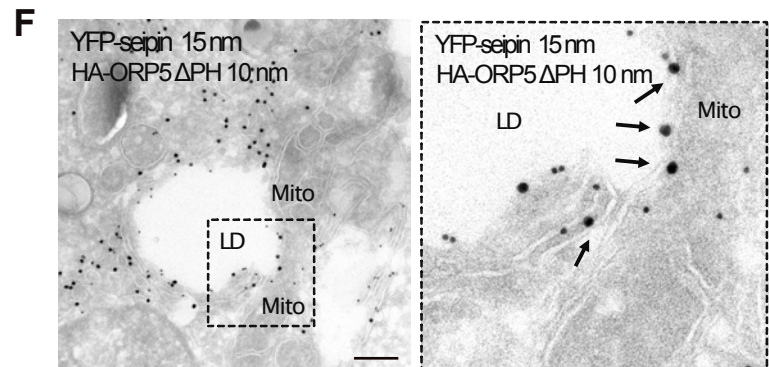
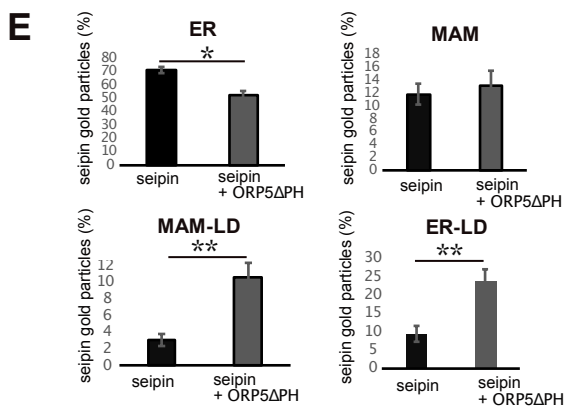
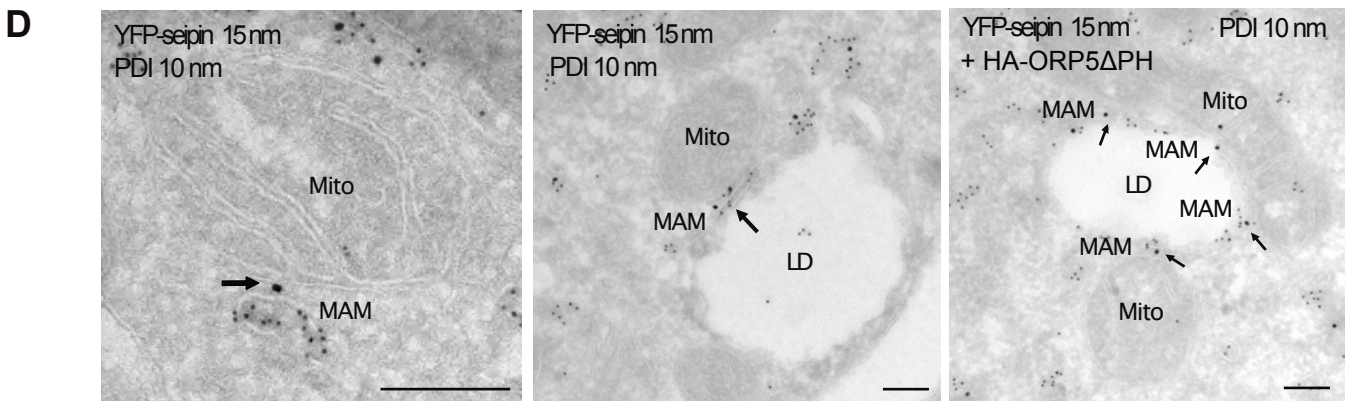
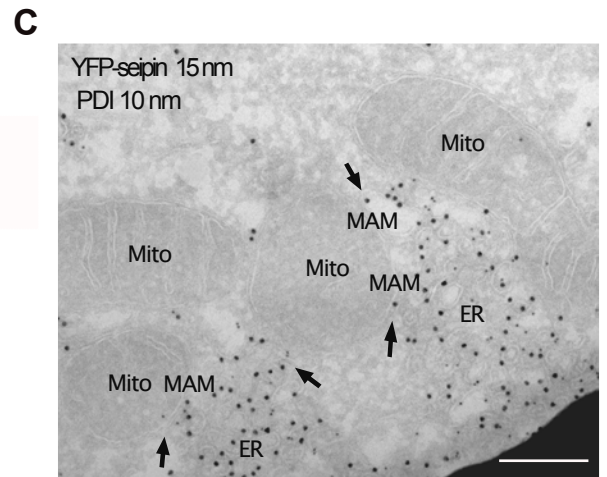
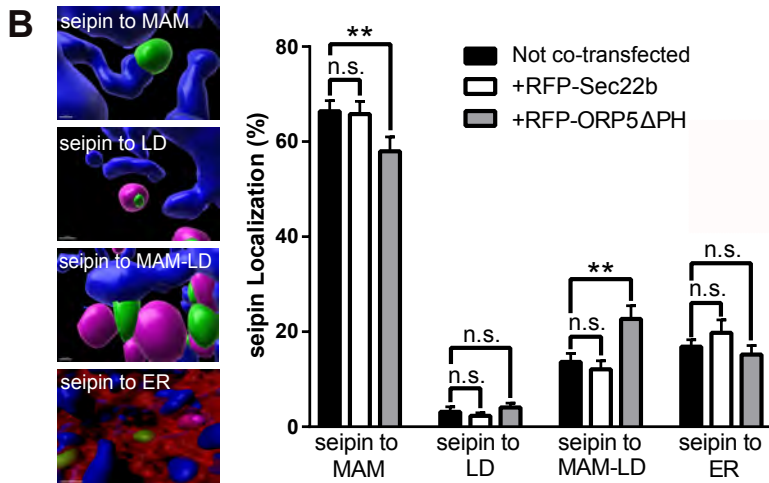
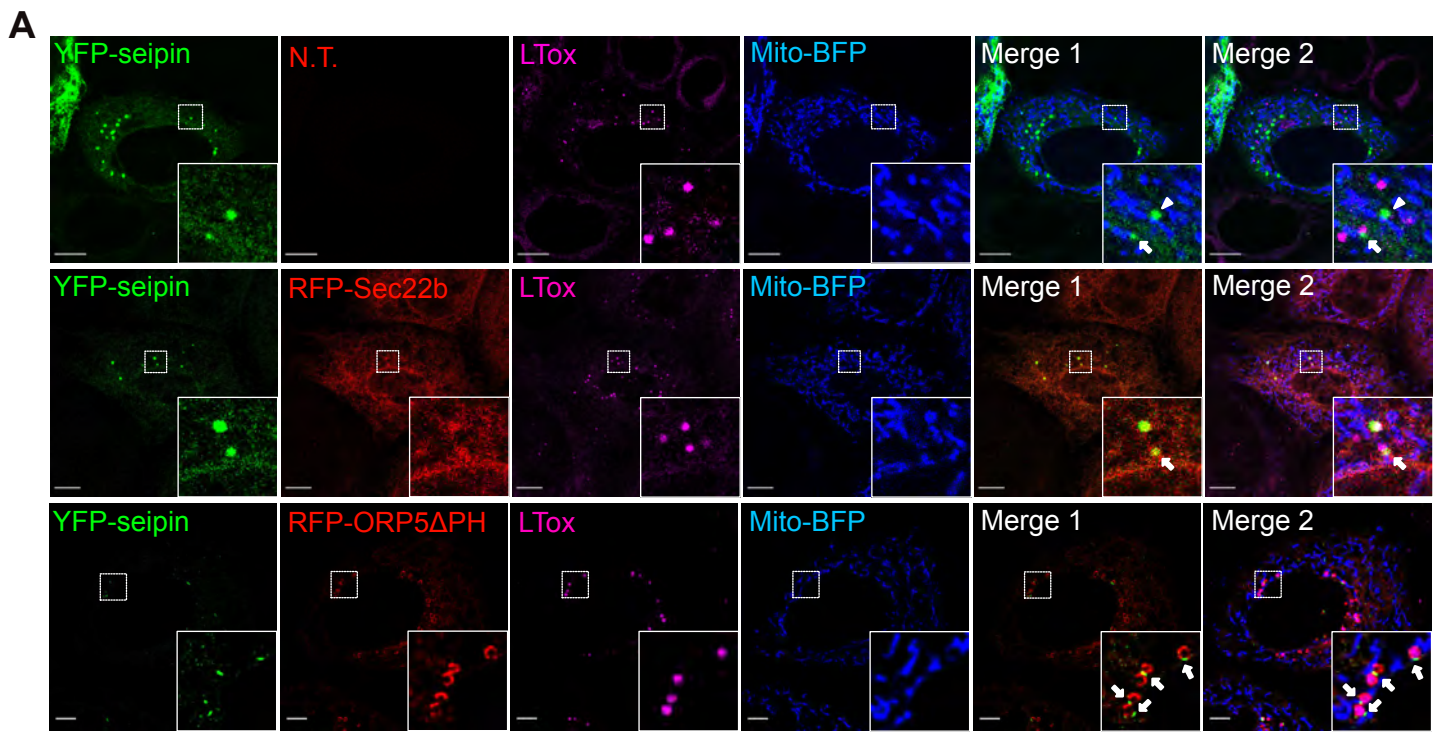


**Figure 5**

**Figure 5. ORP5 localizes to LDs and ER subdomains enriched in phosphatidic acid (PA)**

**A)** Confocal images (single focal plane of HeLa cells expressing EGFP-tagged ORP5A or ORP5 $\Delta$ CC (green), treated with OA (300 $\mu$ M) for 2hr. The mitochondria and the LDs were stained with Mitotracker (red) and LTox (purple) respectively. Arrowhead points ORP5-labeled MAM-LD associated to mitochondria and asterisks marks ORP5 localized to reticular ER. Scale bar = 10  $\mu$ m. **B)** PIP strip overlay assay: PIP strips were incubated with either ORP5-HA CC or ORP8-HA CC or the HA peptide as a negative control and analyzed using the anti-HA antibody. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PtdIns, phosphatidylinositol; PtdIns(3)P; PtdIns(4)P; PtdIns(5)P; PtdIns(3,4)P<sub>2</sub>; PtdIns(3,5)P<sub>2</sub>; PtdIns(4,5)P<sub>2</sub>; PtdIns(3,4,5)P<sub>3</sub>; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate. **C)** Confocal images (single focal plane) of HeLa cells co-expressing EGFP-Opi1p (green) with Mito-BFP (blue) and either EGFP-Sec22b (red) or EGFP-ORP5B (red) or EGFP-ORP5 $\Delta$ PH. The LDs were stained with LTox (purple). Arrowheads points enrichment of Opi1p at Mito-MAM-LD contact sites. Scale bar = 10  $\mu$ m. **D)** 3D reconstruction of cells shown in (C) using IMARIS. Arrows point the MAMs where ORP5B and ORP5 $\Delta$ PH co-localize with Opi1p at Mito-MAM-LD contact sites. Scale bar = 0.5  $\mu$ m.

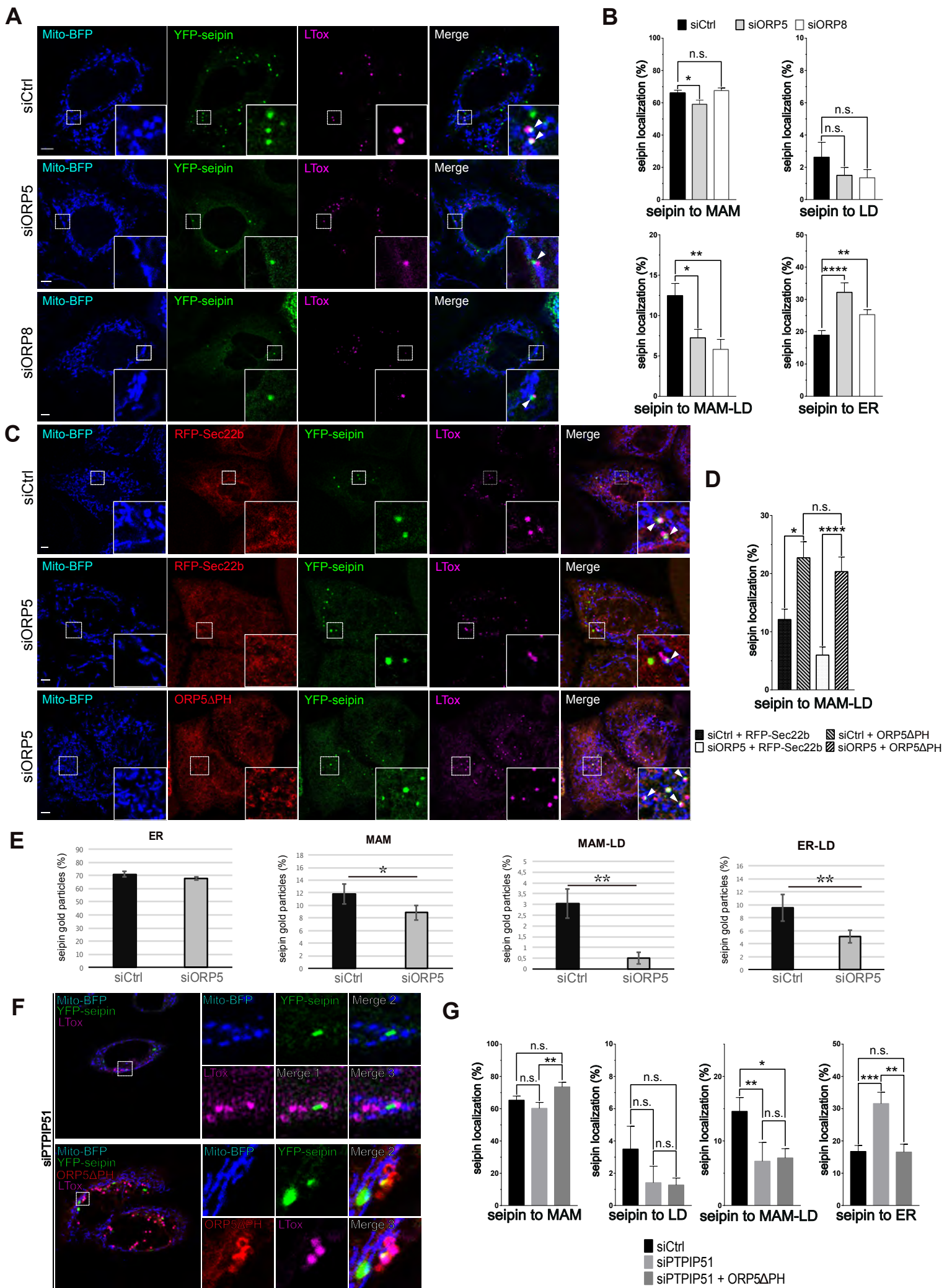




**Figure 6**

**Figure 6. ORP5 over-expression induces an increase of the localization of seipin to MAM-LD contact sites**

**A)** Representative confocal images showing a single focal plane of HeLa cells expressing YFP-seipin (green), Mito-BFP (blue) and Sec22b (red) or ORP5 $\Delta$ PH (red). The LDs were stained LTox (purple). Arrowhead points seipin enrichment at MAM-mitochondria contact sites and arrows mark seipin enrichment at Mito-MAM-LD contact sites. Scale bar = 10  $\mu$ m. **B)** Analysis of the localization of seipin enrichments in HeLa cells expressing seipin alone or in co-expression with Sec22b or ORP5 $\Delta$ PH. Representative 3D reconstruction images of the different categories for the classification of the localization. Data are shown as % mean $\pm$ SEM of cell of n= 56 cells in YFP-seipin (expressed alone), n = 14 cells in YFP-seipin + RFP-Sec22b, and n =40 cells in YFP-seipin + RFP- ORP5 $\Delta$ PH, (\*\* = p<0.01; Wilcoxon-Mann-Whitney test). **C-D)** Representative images of electron micrographs of ultrathin cryosections of HeLa cells transfected with YFP-seipin and immunogold stained with anti-GFP (15 nm gold) to detect seipin and anti-PDI (10 nm gold) to label the ER lumen. Seipin localizes at ER-Mito-LD (arrows). Mito, mitochondria; ER, Endoplasmic Reticulum; MAM, Mitochondria Associated Membranes; LD, Lipid Droplets. Scale bar, 250 nm. **E)** Quantification of the distribution of seipin immunogold particles (15nm). Data is shown as % mean $\pm$ SEM of cell profiles with n=32 (750 gold particles analysed) in seipin individual expression, and n = 50 (940gold particle) in seipin + ORP5 $\Delta$ PH co-overexpression. \*p<0.001, \*\*p<0.0001, unpaired two-tailed *t*-test. **F)** Electron micrographs of ultrathin cryosections of HeLa cells co-transfected with YFP-seipin and HA-ORP5 $\Delta$ PH and immunogold stained with anti-GFP (15 nm gold) to detect seipin and anti-HA (10 nm gold) to detect ORP5. The localization of seipin at MAM-LD is increased when co-expressed with ORP5 $\Delta$ PH (arrows).



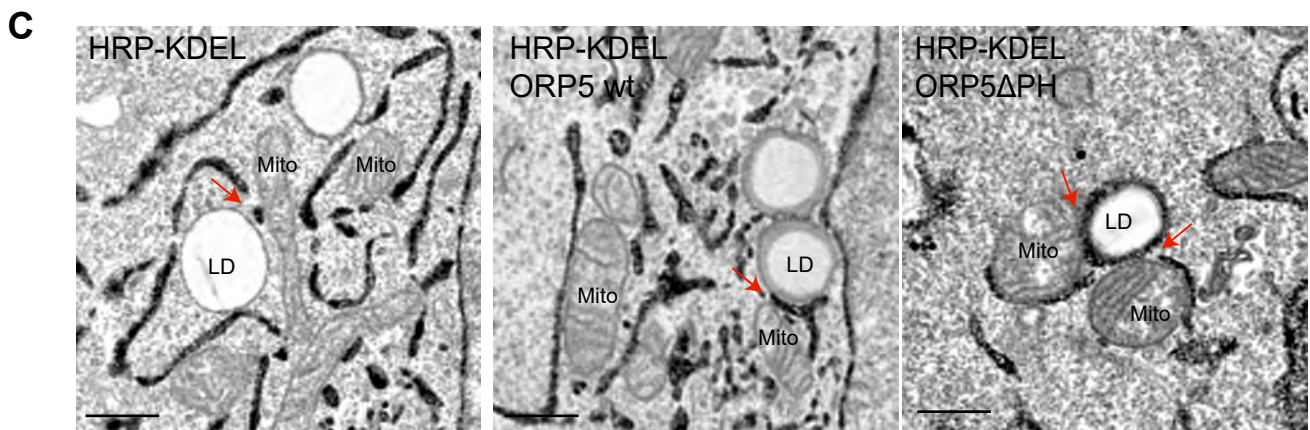
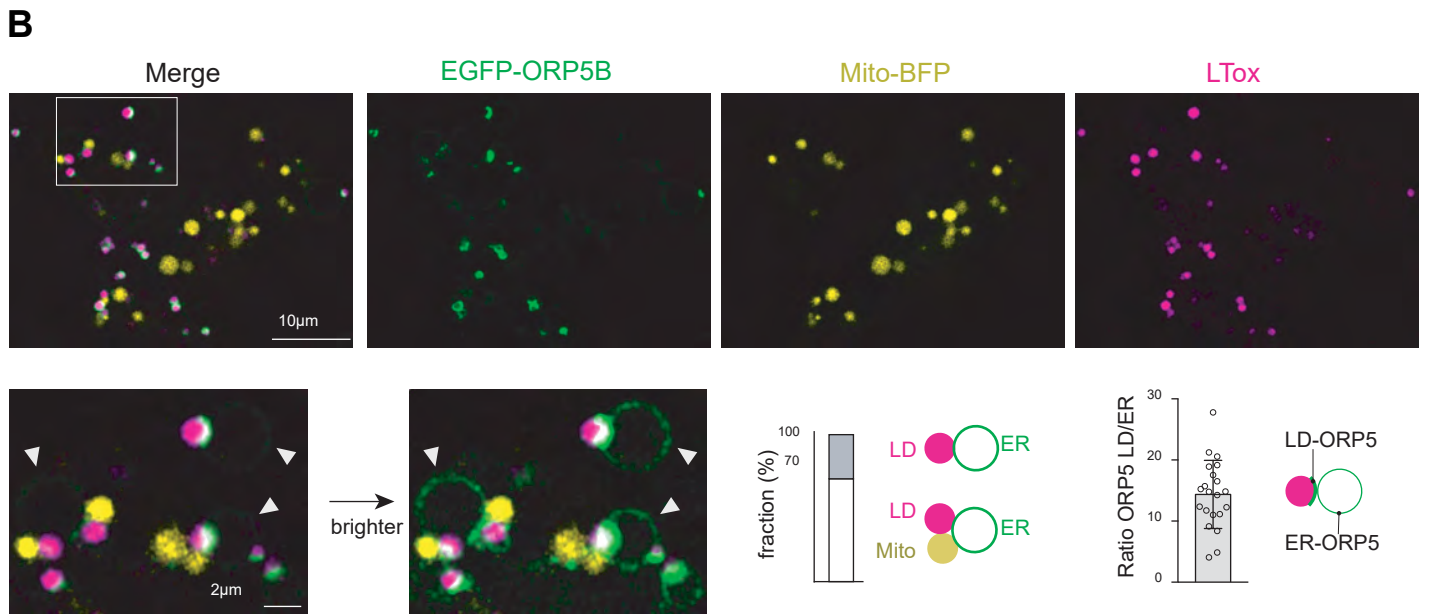
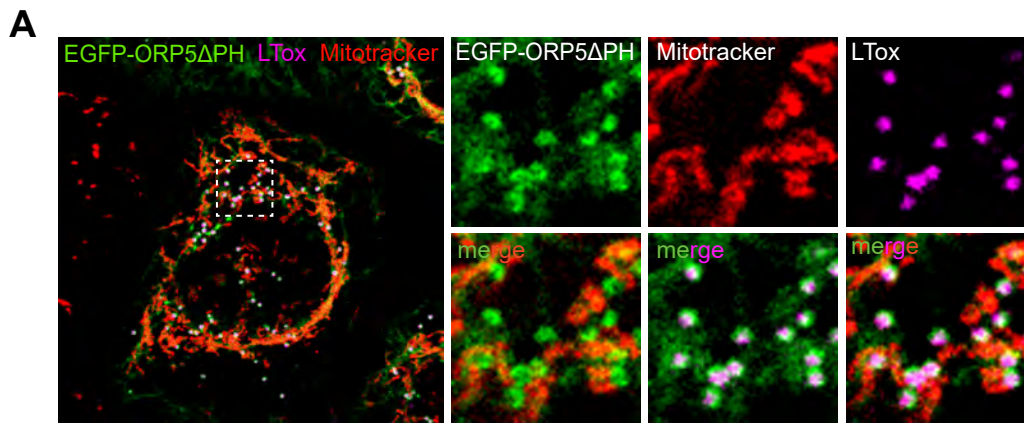
**Figure 7**



**Figure 7. ORP5 affects the localization of seipin in a Mito-MAM contact sites integrity dependent way**

**A)** Representative confocal images (single focal plane) of HeLa cells treated with siCtrl or siORP5 or siORP8 RNA oligos. The cells were then transfected with YFP-seipin (green) and Mito-BFP (blue). The LDs were stained LTox (purple). Arrowheads point seipin enrichment at MAM-LD contact sites. **B)** Analysis of the distribution of seipin enrichments in HeLa cells expressing seipin and treated with either siCtrl, siORP5 or siORP8 interfering RNAs. Data are shown as % mean $\pm$ SEM of cells with n= 64 cells in siCtrl, n = 44 cells in siORP5, and n = 32 cells in siORP8 (\* = p<0.05; \*\* = p<0.01; \*\*\*\* = p <0.0001; Wilcoxon-Mann-Whitney test). **C)** Confocal images (single focal plane) of HeLa cells treated with siCtrl or siORP5 or siORP8 RNA oligos. The cells were then transfected with YFP-seipin (green), Mito-BFP (blue) and either RFP-Sec22b (red) or ORP5 $\Delta$ PH (red). The LDs were stained LTox (purple). Arrowhead points seipin enrichment at MAM-LD contact sites. **D)** Analysis of the distribution of seipin enrichments to MAM-LD contact sites in HeLa cells treated with siCtrl or siORP5 RNA oligos and then co-transfected with seipin and RFP-Sec22b or siRNA-resistant ORP5 $\Delta$ PH. Cells were treated with OA (300 $\mu$ M) for 2h before analysis. Data are shown as % mean $\pm$ SEM of cells. n= 14 cells in siCtrl + Sec22b, n = 17 cells in siORP5 + Sec22b, n = 41 cells in siCtrl + ORP5 $\Delta$ PH rescue and n = 19 cells in siORP5 + ORP5 $\Delta$ PH rescue (\* = p<0.05; \*\*\*\* = p <0.0001; Wilcoxon-Mann-Whitney test). **E)** Quantification of the distribution of the immunogold particles (15nm) staining seipin in HeLa cells treated with siCtrl or siORP5. Data are shown as % mean $\pm$ SEM of cell profiles with n=79 (1500 gold particles analysed) in siCtrl, and n = 64 (1800 gold particle) in siORP5. \* = p<0.001, \*\* = p<0.0001, unpaired two-tailed *t*-test. **F)** Representative confocal images (single focal plane) of HeLa cells treated with siPTPIP51 or siCtrl RNAs. The cells were then transfected with YFP-Seipin (green) alone or in co-expression with ORP5 $\Delta$ PH rescue (red). The LDs were stained with LTox (purple). **G)** Quantification of the distribution of seipin immunogold particles in HeLa cells treated with siCtrl or siPTPIP51 and expressing seipin alone or in co-expression with siRNA-resistant ORP5 $\Delta$ PH. Data are shown as % mean $\pm$ SEM of cells with n= 41 cells in siCtrl, n = 25 cells in siPTPIP51, and n =23 cells in siPTPIP51 + ORP5 $\Delta$ PH (\* = p<0.05; \*\* = p<0.01; \*\*\* = p <0.001; Wilcoxon-Mann-Whitney test).

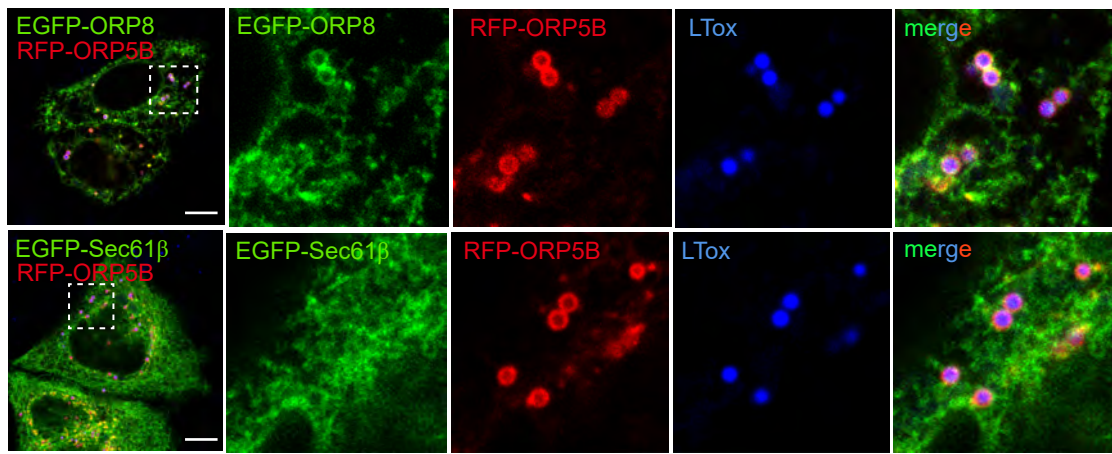
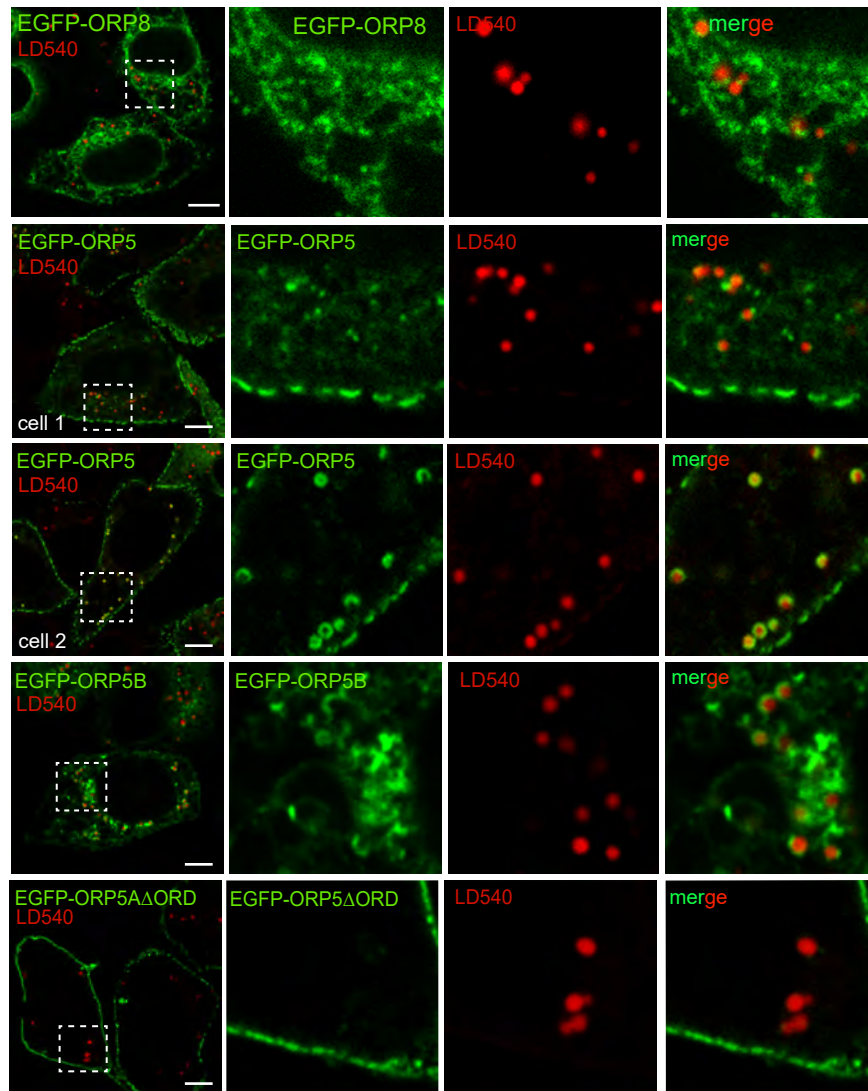
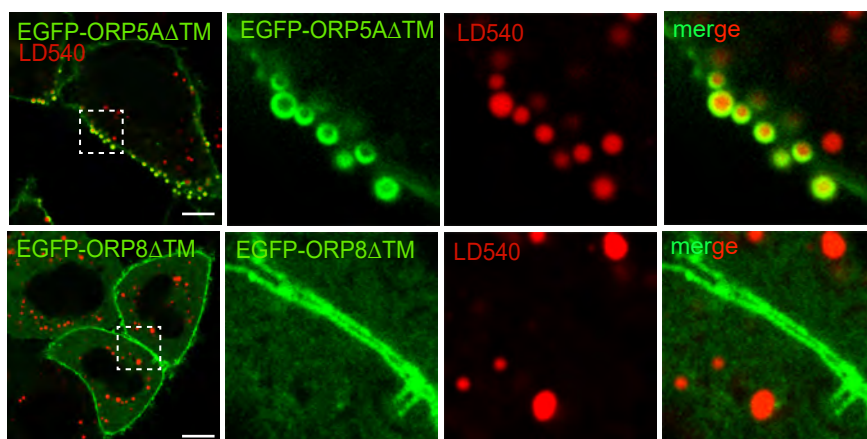
## **SUPPLEMENTARY FIGURES AND LEGENDS**



**Figure S1**

**Figure S1. ORP5 localizes to MAM subdomains in contact with LD**

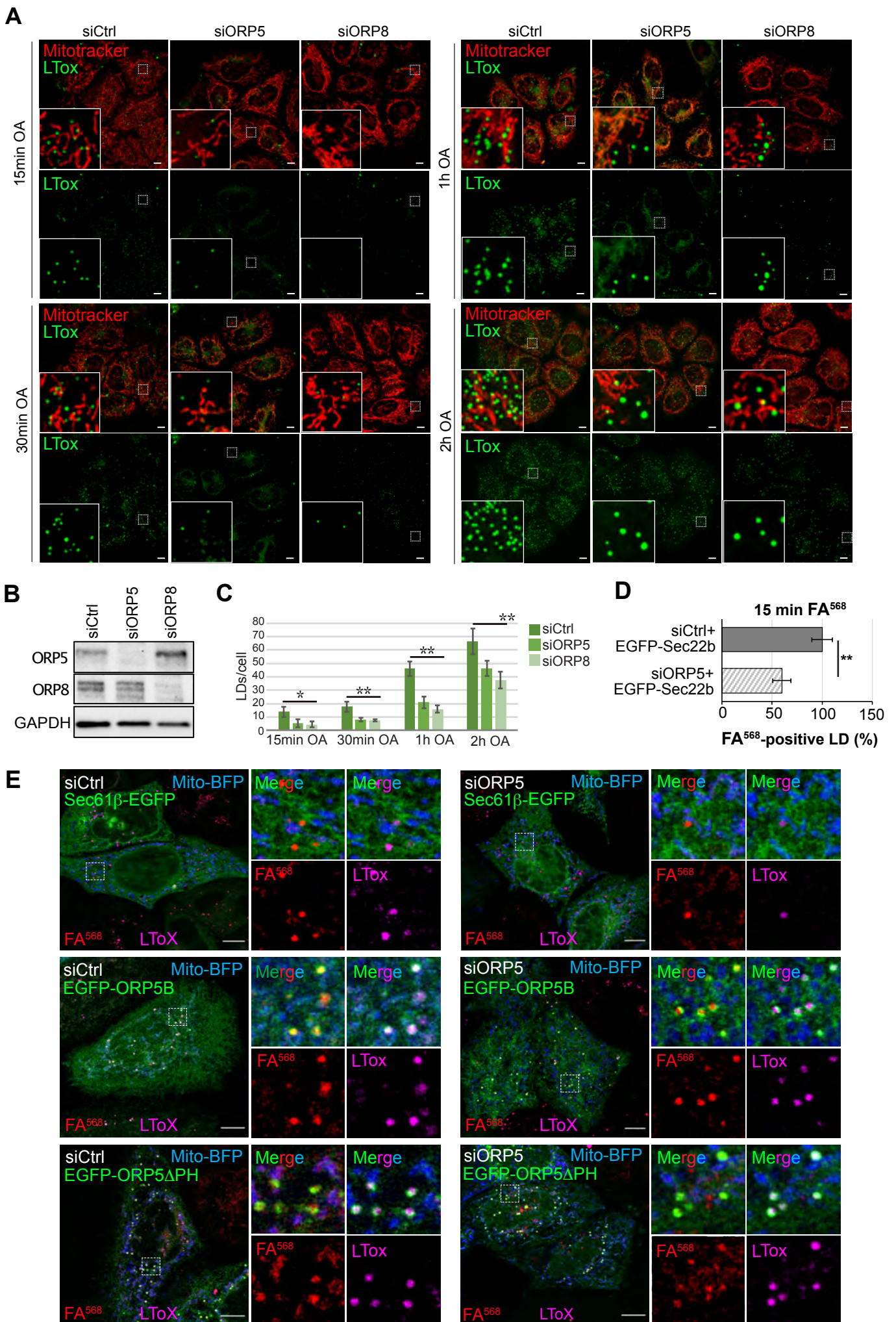
**A)** Representative confocal image (single focal plane) of a HeLa cell expressing ORP5 $\Delta$ PH (green) and treated with Mitotracker (red) and LTox (purple) to label mitochondria and lipid droplets (LD), respectively. Scale bar 10  $\mu$ m. **B)** Live imaging of EGFP-ORP5 (green), Mito-BFP (yellow) and LDs (purple) ten minutes after swelling Huh7 cells. Zoom area is shown below, normal and enhanced EGFP-ORP5 signal to visualize its ER membrane signal (indicated by arrowheads). The bar graphs, from left to right, show (left) the percentage of ER-LD vs. ER-LD contacts events (n=100) and (right) the signal ratio of EGFP-ORP5 intensity at the ER-LD contacts to the ER membrane (n=20), as depicted by the illustrating image. **C)** Electron micrographs of HeLa cells overexpressing HRP-KDEL alone or together with either EGFP-ORP5A or EGFP-ORP5 $\Delta$ PH after OA treatment (300  $\mu$ M for 2hr), and their 3D representation. Scale bar, 250nm

**A****B****C****Figure S2**



**Figure S2. Localization of ORP8 and ORP5 and of their mutants at ER-LD contacts**

**A)** Representative confocal images (single focal plane) of HeLa cells co-expressing either EGFP-ORP8 or RFP-Sec61 $\beta$  (green) and RFP-ORP5B (red) and stained with LTox (blue). Scale bar 10  $\mu$ m. **B)** Confocal images (single focal plane) of HeLa cells expressing EGFP-ORP8, EGFP-ORP5A, EGFP-ORP5B or EGFP-ORP5A $\Delta$ ORD (green) and treated with LD450 (red). Scale bar 10  $\mu$ m. **C)** Confocal images (single focal plane) of HeLa cells expressing EGFP-ORP5A $\Delta$ TM or EGFP-ORP8 $\Delta$ TM (green) and treated with LD450. Scale bar 10  $\mu$ m.



**Figure S3**

**Figure S3. ORP5 and ORP8 depletion impairs LD formation in HeLa cells**

**A)** LD biogenesis time-course. Confocal (single focal plane) images of control (siCtrl), and ORP5 (siORP5) or ORP8 (siORP8) siRNA-treated HeLa cells, delipidated for 72h, and incubated with OA (300  $\mu$ M) for 15 min, 30 min, 1h and 2h. Cells were also stained with Mitotracker (red) and LTox (green). Scale bar, 10  $\mu$ m. **B)** WB analysis showing ORP5, ORP8 and GAPDH levels in protein lysates from Ctrl, ORP5 and ORP8 knockdown HeLa cells. **C)** Quantification of the number of LTox-positive LDs in siCtrl, siORP5 or siORP8 cells in the indicated times after OA delivery. Data are shown as mean $\pm$ SEM of n= 30 cells. \*p<0.01, \*\*p<0.0001, unpaired two-tailed *t*-test **D)** Analysis of FA<sup>568</sup>-positive LD in siCtrl and siORP5 HeLa cells, priorly delipidated for 72h, and then co-transfected with Mito-BFP and EGFP-Sec22b. Data are show as % of siCtrl treated HeLa cells. n= 27 siCtrl and n=24 siORP5. Bar indicated SEM. \*\*p<0.001, unpaired two-tailed *t*-test. **E)** Representative confocal images of control and ORP5 Knockdown delipidated HeLa cells co-expressing Mito-BFP (blue) and Sec61 $\beta$ -EGFP (green), EGF-ORP5B (green), or ORP5 $\Delta$ PH (green), and treated with FA<sup>568</sup> (red, 1  $\mu$ M for 15 min) and LTox (purple). Images represents a single focal plane confocal series, scale bar 10  $\mu$ m.

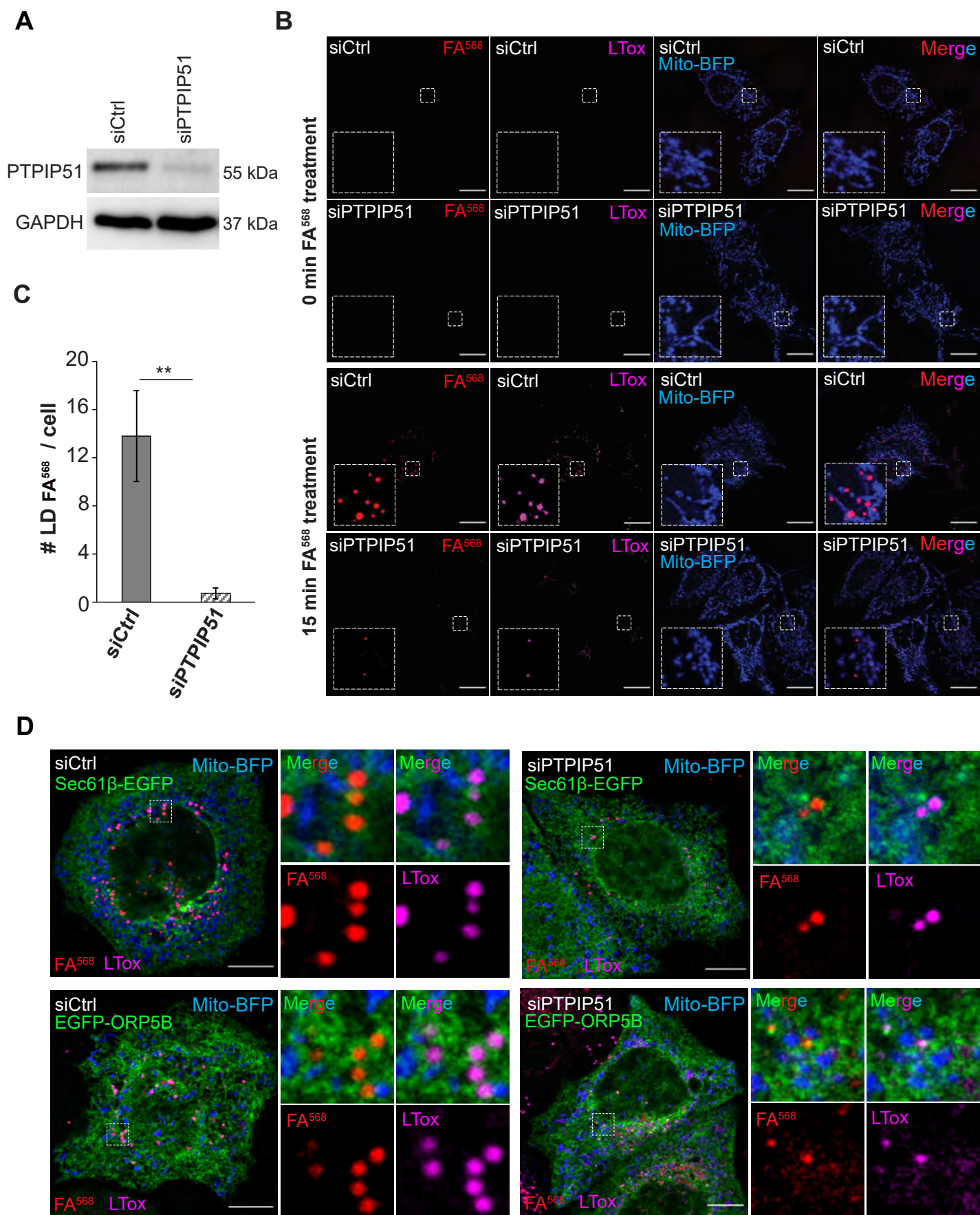
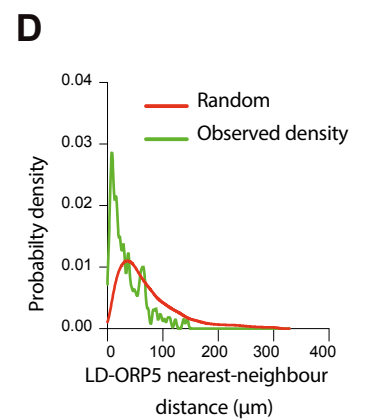
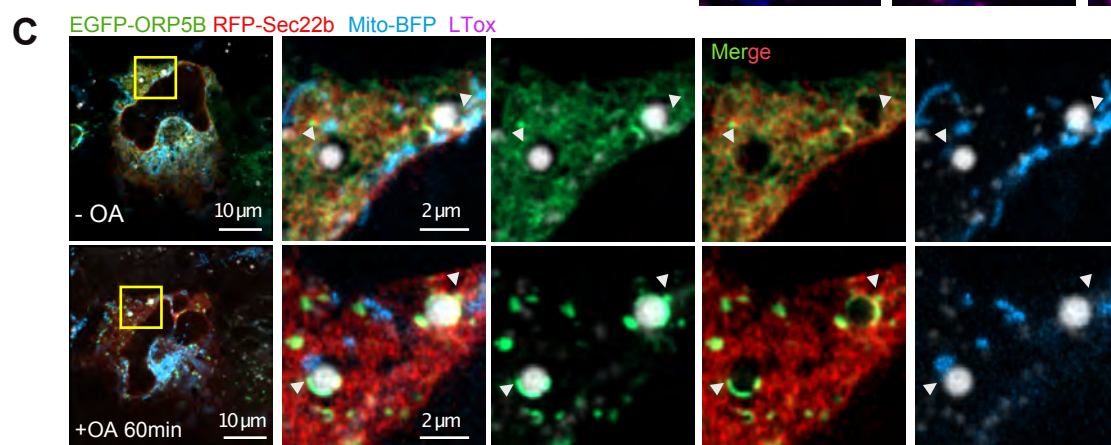
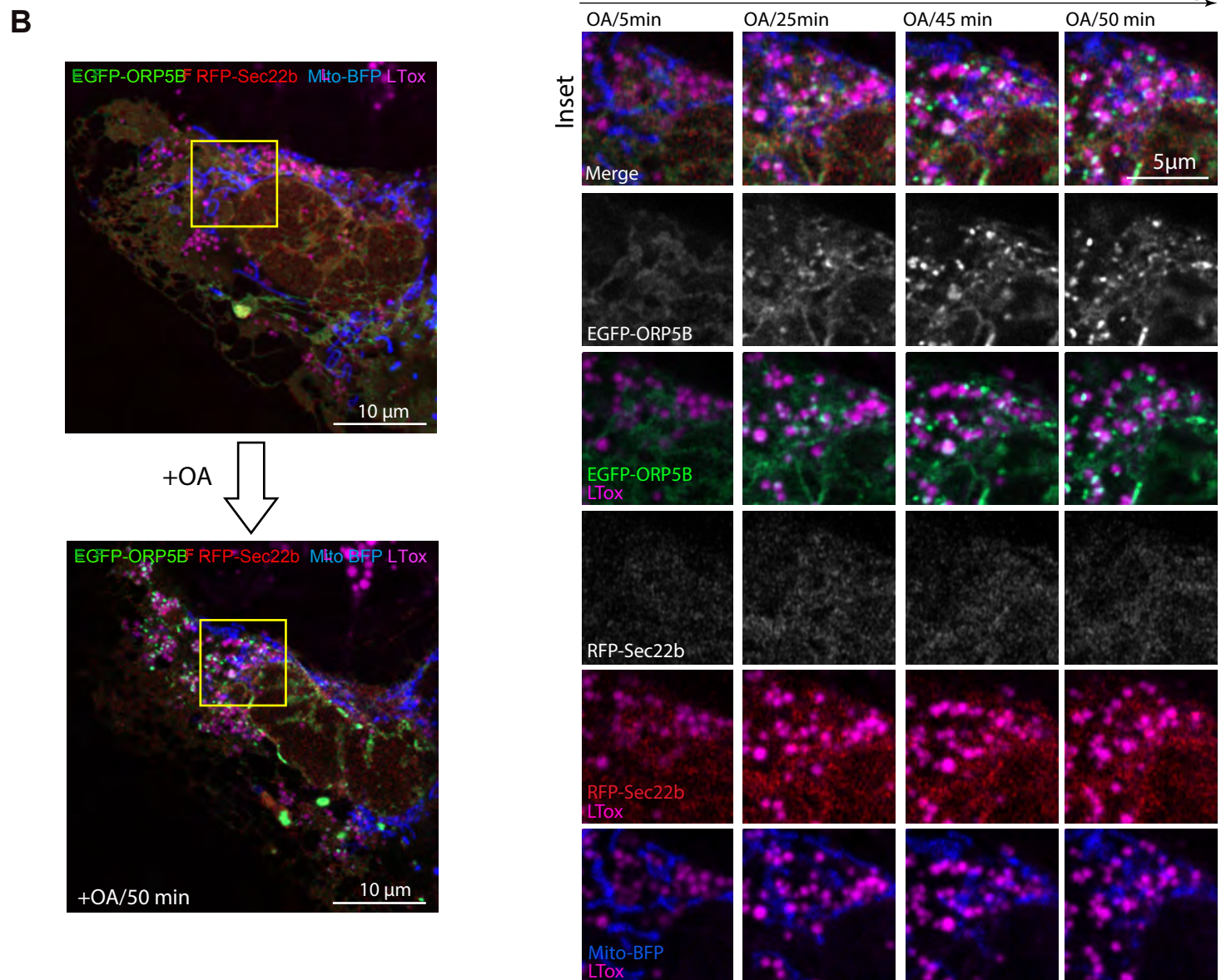
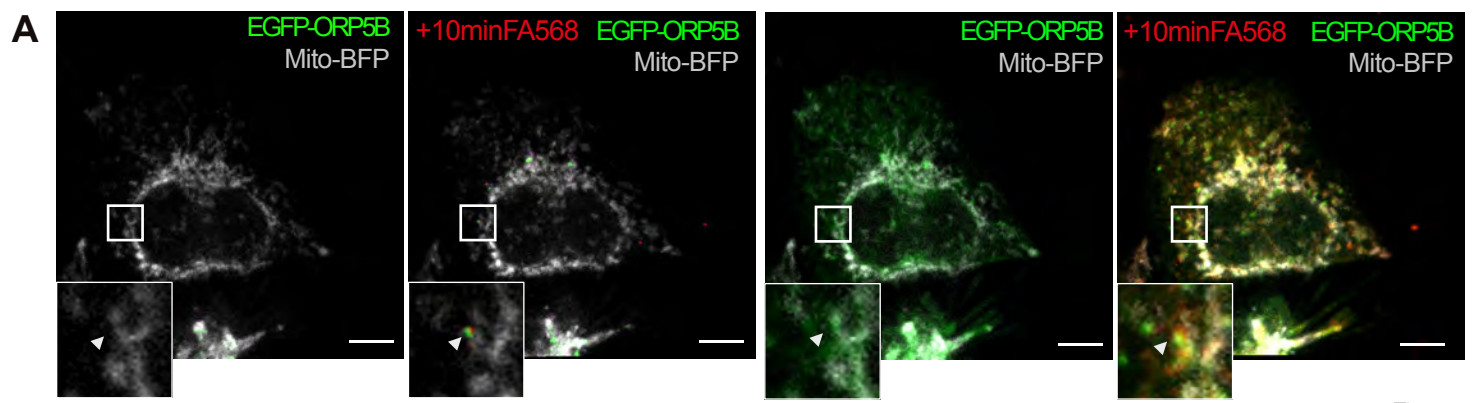


Figure S4

**Figure S4. MAM integrity is required for proper LD Biogenesis**

**A)** Western blot analysis of the expression of PTPIP51 in siCtrl and siPTPIP51 HeLa cells, showing the efficiency of PTPIP51 knockdown. **B)** Confocal (single focal plane) images of control (siCtrl) and PTPIP51 (siPTPIP51) siRNA-treated HeLa cells, delipidated for 72h, and transfected with Mito-BFP (blue). Cells were treated with FA<sup>568</sup> (1  $\mu$ M) for 15 min, and stained with LTox (purple). Scale bar, 10  $\mu$ m. **C)** Analysis of the number of FA<sup>568</sup>-positive LDs in control and PTPIP51 knockdown cells. Data are shown as mean $\pm$ SEM of n=15 in siCtrl and n=16 in siPTPIP51 cells. \*\*\*p<0.001, unpaired two-tailed *t*-test. **D)** Representative confocal images of siCtrl and siPTPIP51 HeLa cells, delipidated for 72hr, and transfected with Mito-BFP (blue) and either Sec61 $\beta$ -EGFP (green) or EGF-ORP5B (green). Cells were loaded with FA<sup>568</sup> (red, 1  $\mu$ M for 15 min) and stained with LTox (purple) before analysis. Images represent a single focal plane of 3D confocal stacks. Scale bar 10  $\mu$ m.



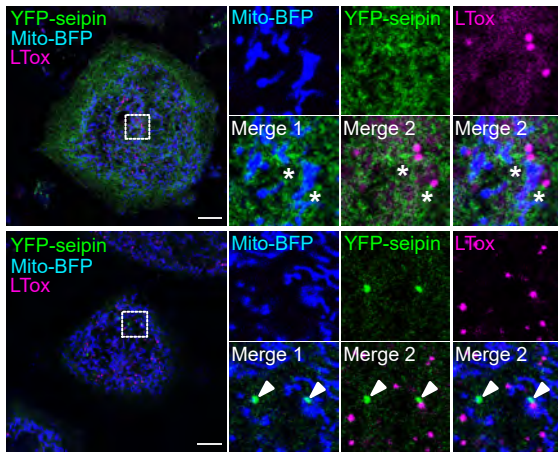
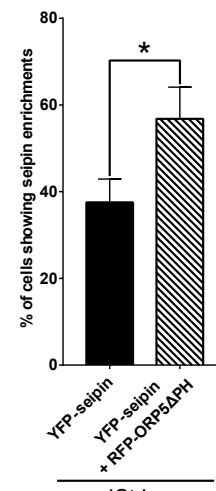
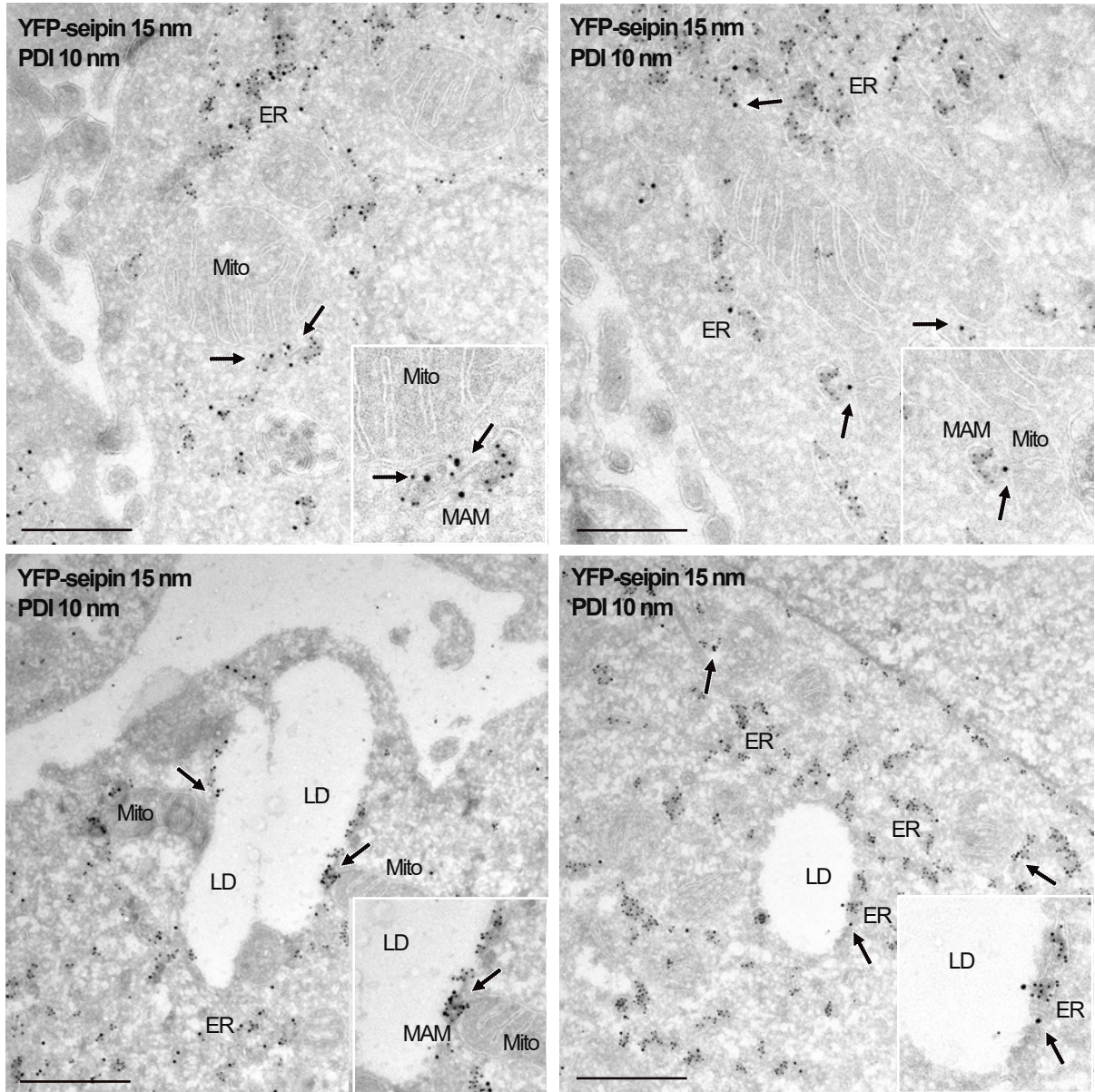
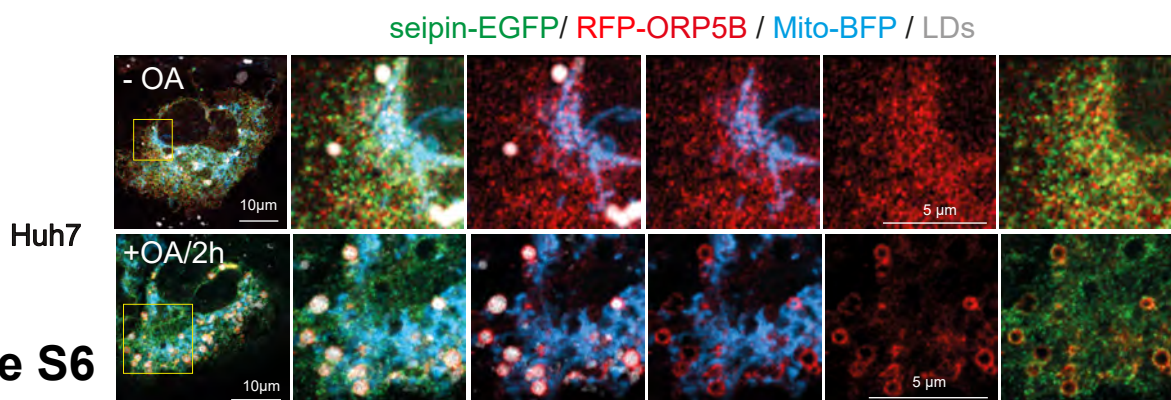


**Figure S5**

**Figure S5. ORP5 redistributes to MAM subdomains where LDs form**

**A)** Spinning video snapshots of HeLa cells expressing EGFP-ORP5B (green) and Mito-BFP (grey). After 2min of acquisition, the cells were treated with FA<sup>568</sup> at 1 $\mu$ M. Left panel shows the cell without brightness and contrast enhancement. Right panel shows the cells with a brightness and contrast treatment to highlight the strongest enrichment of ORP5 and FA<sup>568</sup> at MAM-LD contacts. Arrowheads indicate EGFP-tagged ORP5 Mito-MAM-LD contacts. Scale bar = 1  $\mu$ m. **B)** (related to Figure 4C) Airyscan video snapshots of Huh7 cells expressing EGFP-ORP5B (green), RFP-Sec22b (red) and Mito-BFP (blue). Cells were treated with OA to induce the formation of LDs, stained by LTox (purple). Images were taken every 5 min for 50 min. **C)** Another example depicting the recruitment of ORP5 to pre-existing LDs, with mitochondria. Arrowheads indicate EGFP-tagged ORP5 at Mito-MAM-LD contacts. **D)** Line graph shows the distribution of the nearest-neighbor EGFP-ORP5B puncta to LDs in the images of Huh7 cells treated with OA in (B). The observed probability density largely deviates from randomly distributed LD and puncta EGFP-ORP5B.



**A****B****C****D****Figure S6**



**Figure S6. ORP5 over-expression induces an increase of the localization of seipin to MAM-LD contact sites**

**A)** Confocal images (single focal plane) of HeLa cells expressing YFP-seipin (green) and Mito-BFP (blue). The LDs were stained LTox (purple). Upper panels show a cell with a reticular staining of seipin. Lower panels show a cell with enrichment of YFP-seipin in small “clusters”. Arrowheads show the enrichment of seipin in “clusters” closely opposed to MAM-LD contact sites. Asterisks show presence of seipin at MAM-LD contacts also in cells where it has a reticular distribution. **B)** Quantification of the % of cells showing seipin enrichment at MAM-LD in cells expressing seipin alone or together with ORP5 $\Delta$ PH. Data are shown as % mean $\pm$ SEM of cells. n= 118 cells in siCtrl, n = 121 cells in siCtrl + RFP-ORP5 $\Delta$ PH. **C)** Representative images of electron micrographs of ultrathin cryosections of HeLa cells transfected with YFP-seipin and immunogold stained with anti-GFP (15 nm gold) to detect seipin and anti-PDI (10 nm gold) to label the ER lumen. Arrows points the localization of seipin at different contact sites (Mito-MAM, MAM-LDs and ER-LD). Mito, mitochondria; ER, Endoplasmic Reticulum; MAM, Mitochondria Associated Membranes; LD, Lipid Droplets. Scale bar = 250 nm. **D)** Confocal images (single focal plane) of Huh7 cells expressing seipin-EGFP (green), RFP-ORP5B (red) and Mito-BFP (blue) before and after 2h of OA treatment. The LDs were stained with LTox (white). Scale bar = 10  $\mu$ m.

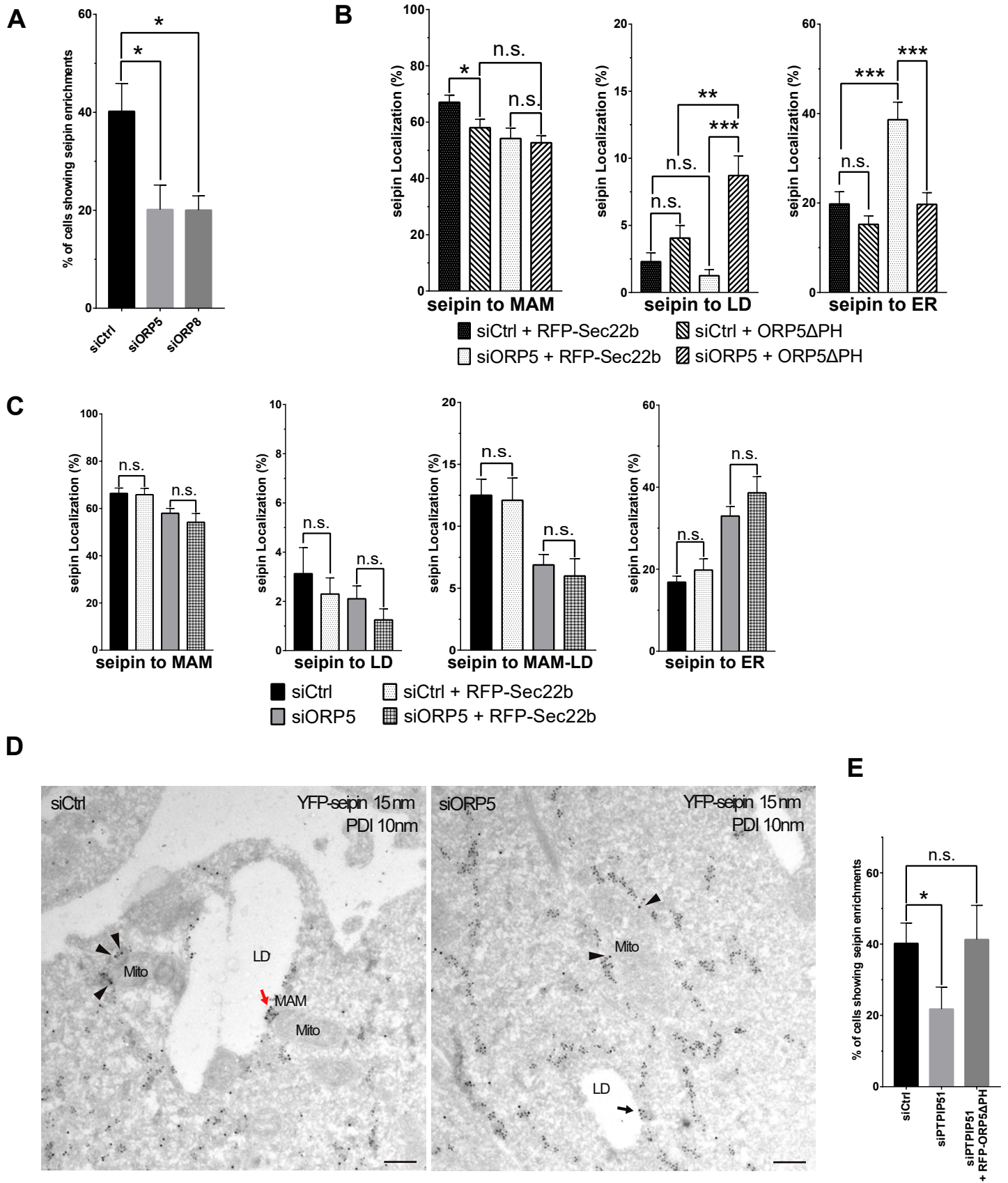


Figure S7

**Figure S7. ORP5 affects the localization of seipin in a Mito-MAM contact sites integrity dependent way**

**A)** Quantification analysis of confocal data showing the percentage of YFP-seipin-expressing siCtrl or siORP5 or siORP8 cells displaying seipin enrichment at MAM-LD. Data are shown as % mean $\pm$ SEM of cell of n=118 cells in siCtrl, n = 80 cells in siORP5 and n = 134 cells in siORP8. **B)** Analysis of seipin localization to the indicated compartments (MAM=Mito-MAM contacts, ER-LD=ER-LD contacts, ER=reticular ER) in siCtrl or siORP5 HeLa cells transfected with YFP-seipin and either RFP-Sec22b or siRNA-resistant ORP5 $\Delta$ PH. Data are shown as % mean $\pm$ SEM of cells. n= 14 cells in siCtrl + Sec22b, n = 17 cells in siORP5 + Sec22b, n = 41 cells in siCtrl + ORP5 $\Delta$ PH rescue and n = 19 cells in siORP5+ ORP5 $\Delta$ PH rescue (\* = p<0.05; \*\* = p<0.01; \*\*\* = p <0.001; Wilcoxon-Mann-Whitney test). **C)** Quantitative analysis of the distribution of seipin enrichments in the indicated compartments (MAM=Mito-MAM contacts, ER-LD=ER-LD contacts, MAM-LD=Mito-MAM-LD contacts, ER=reticular ER) in HeLa cells treated with siCtrl or siORP5 and expressing YFP-seipin alone or together with RFP-Sec22b. Data are shown as % mean $\pm$ SEM of cell of n = 56 cells in siCtrl, n= 14 cells in siCtrl + Sec22b, n = 58 cells in siORP5 and n = 17 cells in siORP5 + Sec22b (n.s. = not significant; Wilcoxon-Mann-Whitney test). **D)** Representative electron micrographs of ultrathin cryosections of HeLa cells transfected with YFP-seipin and immunogold stained with anti-GFP (15 nm gold) to detect seipin and anti-PDI (10 nm gold) to label the ER lumen. Mito, mitochondria; ER, Endoplasmic Reticulum; MAM, Mitochondria Associated Membranes; LD, Lipid Droplets. Scale bar = 250 nm **E)** Quantification of the % of siCtrl or siPTPIP51 cells transfected with YFP-seipin or of siPTPIP51 cells co-transfected with YFP-seipin and RFP-ORP5 $\Delta$ PH, showing seipin enrichment at MAM-LD. Data are shown as % mean $\pm$ SEM of cell of n= 118 cells in siCtrl, n = 91 cells in siPTPIP51 and n =58 cells in siPTPIP51 + RFP-ORP5 $\Delta$ PH.

## **SUPPLEMENTARY MOVIES**

**Movies S1 and S2: ORP5 localizes to ER subdomains where LDs originate (related to Figure 4)**

Time-lapse of live HeLa cell co-expressing fluorescent proteins Mito-BFP (grey) and EGFP-ORP5B (green). After 2 min of acquisition, the cells were treated with FA<sup>568</sup> at 1  $\mu$ M. Movie S1 shows the entire cell and Movie S2 shows a zoom of a region of the HeLa cell.