1   **ORP5/8 AND MIB/MICOS LINK ER-MITOCHONDRIA AND**

2   **INTRAMITOCHONDRIAL CONTACTS FOR NON-VESICULAR TRANSPORT OF**

3   **PHOSPHATIDYLSERINE**

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32   **SUMMARY**

33   Mitochondria are dynamic organelles essential for cell survival whose structural and

34   functional integrity rely on selective and regulated transport of lipids from/to the

35   endoplasmic reticulum (ER) and across the mitochondrial intermembrane space. As they

36   are not connected by vesicular transport, the exchange of lipids between ER and

37   mitochondria occurs at sites of close organelle apposition called membrane contact sites.

38   However, the mechanisms and proteins involved in these processes are only beginning

39   to emerge. Here, we show that the main physiological localization of the lipid transfer

40   proteins ORP5 and ORP8 is at mitochondria-associated ER membranes (MAMs)

41   subdomains, physically linked to the MIB/MICOS complexes that bridge the two

42   mitochondrial membranes. We also show that ORP5/8 mediate non-vesicular transport of

43   phosphatidylserine (PS) lipids from the ER to mitochondria by cooperating with the

44   MIB/MICOS complexes. Overall our study reveals a novel physical and functional link

45   between ER-mitochondria contacts involved in lipid transfer and intra-mitochondrial

46   membranes contacts maintained by the MIB/MICOS complexes.

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48   **KEYWORDS**

49   Membrane contact sites, MAM, ER, mitochondria, ORP, phosphatidylserine.

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50   **INTRODUCTION**

51   Vesicular trafficking is the major pathway for transport of proteins and lipids between

52   membranes. However, an alternative route, which is vesicle-independent, occurs at

53   regions of close inter-organelle membrane proximity (within less than 30 nm) also called

54   membrane contact sites (Scorrano, De Matteis et al., 2019). This route is particularly

55   important to preserve membrane composition, integrity and identity of intracellular

56   organelles such as mitochondria that are largely excluded from the classical vesicle-

57   mediated trafficking pathway. Like other organelles, mitochondria can be closely

58   associated with the endoplasmic reticulum (ER), the major site of lipid synthesis and the

59   major intracellular calcium (Ca^{2+}) store. ER membrane subdomains closely apposed to

60   mitochondria are called mitochondria-associated ER membranes (MAMs) and they

61   facilitate the exchange of Ca^{2+} and lipids between the two organelles (Herrera-Cruz &

62   Simmen, 2017, Tatsuta, Scharwey et al., 2014, Vance, 2014).

63   Mitochondria are involved in a plethora of cellular processes including energy

64   production, lipid metabolism, Ca^{2+} homeostasis and apoptosis. To fulfill their numerous

65   functions, mitochondria need to maintain a defined membrane composition by receiving

66   essential lipids and lipid precursors from the ER through membrane contact sites (Acoba,

67   Senoo et al., 2020, Giordano, 2018, Vance & Tasseva, 2013).

68   Increasing lines of evidence suggest that lipid transfer proteins (LTPs) play a major

69   role in regulating the lipid composition of membranous organelles by facilitating non-

70   vesicular lipid transport at membrane contact sites. In recent years, several tethering

71   complexes with lipid transfer activity have been identified at membrane contact sites

72   between the ER and other intracellular organelles as well as the plasma membrane (PM)

73   in yeast and mammalian cells (Wong, Gatta et al., 2019). However, our knowledge of how

74   lipids are exchanged at ER-mitochondria membrane contact sites remains rudimentary,

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75 and the LTPs that localize and function at these sites are just starting to be discovered.

76 The best-studied lipid transfer/tethering complex at ER-mitochondria contact sites is the

77 yeast ER-mitochondria encounter structure (ERMES) (Kornmann et al 2009, Lang et al

78 2015) that bridges the ER and the mitochondrial membranes and also facilitates the

79 exchange of phospholipids (in particular phosphatidylcholine, PC) between them (ref).

80 In metazoans, very little is known on how lipids are exchanged at ER-mitochondria

81 membrane contact sites and about the proteins involved in this process. Some

82 tethering proteins at mammalian ER-mitochondria contact sites have emerged in the

83 recent years, such as VDAC-GRP75-IP3R and PTPIP51-VAPB complexes (Gatta and

84 Levine 2017). A recent study has proposed that PTPIP51 could regulate cardiolipin

85 levels by transferring its precursor phosphatidic acid (PA) to the mitochondria via its

86 TPR domain (Yeo, Park et al., 2021). However, further work is required to clarify the

87 lipid transfer ability of the PTPIP51 TPR domain (Francesca Giordano, 2021). Also,

88 recently, mammalian LTPs with tethering function, such as VPS13A or Pdzd8 (the

89 latter being proposed as a paralog of the ERMES subunit Mmm1(Wideman, Balacco

90 et al., 2018)), were shown to localize at membrane contact sites, including those

91 between ER and mitochondria, where they regulate membrane tethering and, in the

92 case of Pdzd8, mitochondrial Ca^{2+} uptake (Hirabayashi, Kwon et al., 2017, Kumar,

93 Leonzino et al., 2018). However, a direct involvement of these proteins in non-vesicular

94 lipid transport between ER and mitochondrial membranes remains to be proved.

95 The Oxysterol binding protein (OSBP)-related proteins constitute a large family of

96 LTPs conserved from yeast (Osh) to humans (ORP) and localized to different

97 subcellular sites, shown in several cases to be membrane contact sites. A common

98 feature of all ORPs is the presence of an OSBP-related lipid-binding/transfer (ORD)

99 domain. Most ORP proteins contain a two phenylalanines (FF) in an acidic tract

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100   (FFAT)-motif that binds ER-localized VAP proteins and a pleckstrin homology (PH)

101   domain that interacts with lipids or proteins in distinct non-ER organelle membranes.

102   Two members of this family, ORP5 and ORP8, do not contain an FFAT motif but are

103   directly anchored to the ER through a C-terminal transmembrane (TM) segment

104   (Olkkonen, 2015).

105   ORP5 and ORP8 have been previously shown to localize at ER-PM contact sites

106   where they transfer phosphatidylserine (PS) from the cortical ER to the PM, in counter-

107   exchange with the phosphoinositides Phosphatidylinositol-4-phosphate (PI4P) and

108   Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (Chung et al. 2015; Ghai et al, 2017). We

109   have recently shown that ORP5 and ORP8 are also present in the MAM and play a

110   key role in maintaining mitochondrial integrity (Galmes, Houcine et al., 2016). We and

111   others have also shown that ORP5/8 form a protein complex in the cell (Chung, Torta

112   et al., 2015, Galmes et al., 2016). However, ORP5 and ORP8, when overexpressed,

113   display a different distribution within MCS. In particular, overexpression of ORP5

114   greatly expands ER-PM contacts (Chung et al., 2015, Galmes et al., 2016), resulting

115   in an accumulation of ORP5 at these sites, while overexpressed ORP8 is largely

116   retained in the reticular ER. As all the studies on ORP5 and ORP8 so far have

117   employed their individual overexpression, the endogenous sites where ORP5 and

118   ORP8 interact and function as a complex are still unknown.

119   Interestingly, transport of PS is a key event occurring at ER-mitochondria contact

120   sites. Newly synthesized PS, by the ER-localized PS-Synthase 1 (PSS1), is shuttled from

121   the ER to the outer mitochondrial membrane (OMM) and from OMM to inner mitochondrial

122   membrane (IMM) where it is rapidly converted to phosphatidylethanolamine (PE) by the

123   PS-decarboxylase enzyme PISD (Tamura, Kawano et al., 2020, Vance, 2014, Vance &

124   Tasseva, 2013). At the IMM, PE plays crucial roles in maintaining mitochondrial tubular

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125   morphology and therefore mitochondrial respiratory functions (Joshi, Thompson et al.,

126   2012, Steenbergen, Nanowski et al., 2005). Regardless of extensive studies on PS

127   transport between ER and mitochondria since its first discovery more than 20 years ago

128   (Vance, 1990), the underlying mechanisms and proteins involved are still elusive.

129   Membrane contact sites exist also between the OMM and the IMM and are mediated

130   by the Mitochondrial Intermembrane space Bridging (MIB) and Mitochondrial Contact

131   sites and Cristae junction Organizing System (MICOS) complexes. The MICOS complex

132   is a multi-subunit complex preferentially located at Cristae Junctions (CJ), tubular

133   structures that connect the IMM to the cristae, and it is necessary for CJ formation, cristae

134   morphology and mitochondria function (Harner, Korner et al., 2011, Huynen,

135   Muhlmeister et al., 2016, Ott, Dorsch et al., 2015, Wollweber, von der Malsburg et al.,

136   2017). The integral IMM protein Mic60 is the central component of the MICOS complex

137   and carries a large domain exposed to the mitochondria intermembrane space (IMS)

138   that interacts with the OMM Sorting and Assembly Machinery (SAM) to form the MIB

139   complex (Friedman, Mourier et al., 2015, Guarani, McNeill et al., 2015). The SAM

140   complex is constituted of SAM50 (a pore-forming β-barrel protein), metaxin1 and 2,

141   and is involved in the membrane insertion and assembly of mitochondrial β-barrel

142   proteins (Hohr, Lindau et al., 2018, Kozjak, Wiedemann et al., 2003, Kozjak-Pavlovic,

143   Ross et al., 2007). However, whether and how OMM-IMM contact sites are linked to

144   ER-mitochondria contacts in mammalian cells is still largely unknown.

145   Here we uncover for the first time the endogenous localization of ORP5 and ORP8,

146   revealing that the major site of their interaction in physiological conditions are the

147   MAMs. We also show that the ER subdomains where ORP5 and ORP8 reside are

148   physically connected to the intra-mitochondrial membrane contacts bridged by the

149   MIB/MICOS complexes at cristae junctions. We then show that ORP5/8 cooperate with

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150   SAM50 and Mic60, key components of the MIB/MICOS complex, to mediate PS

151   transport from the ER to the mitochondrial membranes at ER-mitochondria contact

152   sites in mammalian cells, and consequently the synthesis of mitochondrial PE.

153   Our findings reveal a novel tripartite association between the ER and the two

154   mitochondrial membranes that links lipid transfer across these membranes, cristae

155   biogenesis and consequently mitochondria function.

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157   **RESULTS**

158   **ER-mitochondria contact sites are the main localization of the ORP5-ORP8**

159   **complex**

160   The localization of ORP5 and ORP8 at endogenous level is still unknown. We thus

161   investigated their endogenous localization by immunofluorescence (IF) using antibodies

162   against ORP5 and ORP8 proteins. First, we validated the specificity of these antibodies

163   in cells overexpressing ORP5 or ORP8 proteins fused with a similar fluorescent tag

164   (EGFP-ORP5 or EGFP-ORP8) and found that ORP5 and ORP8 signals detected using

165   these antibodies co-localized with the overexpressed proteins (Fig. S1a-b). Then, we

166   analyzed ORP5 and ORP8 endogenous localization in control HeLa cells and in cells

167   where ORP5 and ORP8 were downregulated by RNAi and whose mitochondria were

168   labeled by MitoTracker. We found a strong decrease in ORP5 and ORP8 IF labeling upon

169   their knockdown (KD) (Fig. 1a, 1c, S1f), whose efficiency was confirmed by western

170   blotting (WB) (Fig. 1b, S1c-f), validating the specificity of the used antibodies. ORP5 and

171   ORP8-positive compartments in control conditions overlapped with the ER protein RFP-

172   Sec22b, confirming endogenous ORP5 and ORP8 localization to the ER and further

173   validating the specificity of these antibodies (Fig. S2a). Interestingly, the majority of

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174   endogenous ORP5 and ORP8 co-localized to subcellular compartments in close proximity

175   to mitochondria (Fig. 1a, 1d, 1e-f).

176   We then sought to analyze ORP5 and ORP8 localization when co-overexpressed (at

177   similar levels) by co-transfecting HA-ORP5 and EGFP-ORP8 in HeLa cells and comparing

178   their localization with the individually expressed EGFP-ORP5 and EGFP-ORP8 by

179   confocal microscopy. When expressed alone, EGFP-ORP5 localizes to ER in contact with

180   mitochondria, but also strongly increases ER-PM contact sites where it relocates, while it

181   localizes very little in the reticular ER (Fig. 1g, S2b and (Galmes et al., 2016)). Instead,

182   EGFP-ORP8, when expressed alone, localizes mostly to ER-mitochondria contacts and

183   to reticular ER, with only a minor pool at cortical ER, as it does not increase ER-PM contact

184   sites (Fig. 1g, S2b). Remarkably, even if the individually overexpressed ORP5 and ORP8

185   were differently distributed among MCS, their localization at ER-mitochondria contacts

186   was higher as compared to a general ER protein, such as Sec22b (Fig. 1h). Moreover,

187   and interestingly, when expressed together, ORP5 and ORP8 equally redistributed and

188   co-localized to cortical ER, reticular ER and ER-mitochondria contacts (Fig. 1g, S2b). In

189   particular, their localization to ER-mitochondria contacts was higher as compared to when

190   expressed individually (Fig. 1h). Also, the co-localization of co-overexpressed ORP5 and

191   ORP8 was comparable to the co-localization of the endogenous proteins, as revealed by

192   the high Pearson's correlation coefficient that was in both cases close to 1 (Fig. 1f). These

193   data indicate that co-expression of ORP5 and ORP8 mimics the physiological localization

194   of these proteins as a complex, when the expression levels of one of the two proteins are

195   not highly enriched as compared to the other.

196   To further quantify ORP5 and ORP8 co-localization and interaction at ER-

197   mitochondria contact sites in co-overexpression and endogenous conditions we used

198   Duolink-Proximity Ligation Assay (PLA) coupled with staining of mitochondria

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199   (MitoTracker) and confocal microscopy. PLA signals corresponding to ORP5-ORP8

200   interaction were observed throughout the cell in both endogenous and co-overexpression

201   (HA-ORP5 and 3XFLAG-ORP8) conditions (Fig. 2a). The specificity of this assay and of

202   the antibodies used was confirmed by the strong decrease in PLA signals for endogenous

203   ORP5-ORP8 interaction in cells with ORP5 and ORP8 knocked down (Fig. 2b, S3a).

204   Likewise, a significant increase in ORP5-8 PLA signals was induced by the

205   overexpression of these proteins (Fig. 2b)

206   Close association of PLA spots to mitochondria, indicating localization at ER-

207   mitochondria contact sites, was measured after segmentation of the mitochondrial

208   network by Imaris (Fig. 2a, right panel; S3b). Interestingly, the majority of ORP5-8 PLA

209   signals localized at ER-mitochondria contact sites (52% of endogenous ORP5-8 and 50%

210   of co-overexpressed ORP5-8) (Fig. 2a, 2c). The localization of ORP5-8 PLA signals to

211   ER-PM contact sites was analyzed in HeLa cells transfected with RFP-PH-PLCδ, to stain

212   the PM, and Mito-BFP, to label mitochondria. However, only a minor pool of ORP5-8 PLA

213   spots was found in contact with the PM (4% of endogenous ORP5-8 and 5% of co-

214   overexpressed ORP5-8) (Fig. 2d-f). The localization of ORP5-ORP8 PLA spots to the ER,

215   including MAMs, was confirmed in cells co-expressing the ER protein Sec22b and the

216   mitochondrial-targeted Mito-BFP (Fig. S3c).

217   Overall these data reveal for the first time that the main sites where ORP5 and ORP8

218   localize and interact in endogenous physiological conditions are the ER-mitochondria

219   contact sites, and not the ER-PM contacts.

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221   **ORP5/8 physically interact with the mitochondrial intermembrane space**

222   **bridging (MIB) complex facing cristae junctions**

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223   To investigate whether ORP5/8 localize to specific ER-mitochondria contact

224   subdomains we performed a morphological analysis of ORP5 localization by immuno-

225   EM (IEM) on ultrathin cryosections from HeLa cells transfected with HA-ORP5 or

226   EGFP-ORP5 (as endogenous ORP5/8 levels are too low to be detected by IEM). We

227   previously reported that about 20% of ORP5 or ORP8 gold particles were associated

228   to ER-mitochondria contact sites when individually expressed (Galmes et al., 2016).

229   The advantage of analyzing ORP5 localization is its preferential localization to contact

230   sites (20% at ER-mitochondria contacts and 60% ER-PM contacts), when expressed

231   alone, as compared to ORP8, which remains also largely present within the reticular

232   ER (60% of ORP8 vs 20% of ORP5) (Galmes et al., 2016). Interestingly, the majority

233   of ORP5 gold particles at ER-mitochondria contact sites was found to localize to ER

234   elements in a very close proximity (86% within 0-100 nm distances, 50% of which

235   within 50 nm) to the CJ (arrow, Fig. 3a-b), tubular structures that connect the IMM to

236   the cristae. To exclude that ORP5 localization near CJ is not a consequence of its

237   distribution throughout the ER membranes, we sought to determine if other ER proteins

238   have a similar frequency of proximity to CJ. Thus, we compared ORP5 localization to

239   Sec61β, an ER protein present in ER elements widely distributed throughout the cells

240   and very little at ER-mitochondria contacts (Galmes et al., 2016). Co-immunolabeling

241   of EGFP-ORP5 or EGFP-Sec61β and the luminal ER protein disulfide isomerase (PDI)

242   confirmed ORP5 localization to ER elements close to CJ (arrow, Fig. 3c) but not of

243   Sec61β, the bulk of which localized on ER membranes distant from the CJ (0% within

244   0-100nm and 69% >200nm distance) even when close to mitochondria (Fig. 3b and

245   arrowheads in Fig. 3c). To determine whether other contact sites proteins would be

246   similarly enriched at CJ we analyzed the localization of PTPIP51, a mitochondrial

247   tether known to localize at ER-mitochondria contact sites (Stoica, De Vos et al., 2014)

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248   and also a binding partner of ORP5/8 (Galmes et al., 2016). A small pool of PTPIP51-

249   HA could be detected near CJ where they colocalized with EGFP-ORP8 or EGFP-

250   ORP5. However, the majority of PTPIP51 randomly distributed throughout the

251   mitochondrial surface (Fig. 3d-e). Hence, our results support the conclusion that

252   ORP5/8 specifically localize to ER-mitochondria contact sites closely associated to CJ.

253   Interestingly, in yeast, CJ were shown to be closely associated to OMM-IMM contact

254   sites tethered by the MICOS complex (Harner et al., 2011). IEM analysis using Mic60-

255   EGFP, an EGFP-tagged construct of the human orthologue of the central component

256   of the MICOS complex, confirmed that human Mic60, similarly to its yeast orthologue,

257   preferentially localizes to the IMM in close proximity of CJ and in the cristae that arise

258   from them (arrow, Fig. 3a). These results suggest that ER-mitochondria contact sites

259   where ORP5/8 localize could be physically connected to the intra-mitochondrial

260   membrane contact sites near CJ.

261   To identify binding partners of ORP5/8 at ER-mitochondria contact sites we carried

262   out a MS-analysis on GFP-pull downs from cells expressing EGFP-ORP5, EGFP-

263   ORP5ΔPH (an ORP5 variant lacking the PM-targeting PH domain that is localized at

264   ER-mitochondria but not at ER-PM contact sites) or EGFP alone as a control (Fig. 4a,

265   S4a). As expected, the highest hit detected in both EGFP-ORP5 and EGFP-ORP5ΔPH

266   pull-downs was ORP8. In accord to our previous study (Galmes et al., 2016), the

267   mitochondrial protein PTPIP51 was also detected in the mass spectrometry analysis.

268   Interestingly, several new outer mitochondrial membrane proteins (listed in Fig. 4a)

269   were also found as major hits. Among these proteins, the MIB component SAM50 and

270   the MICOS central subunit Mic60, binding partner of SAM50 at OMM-IMM contact sites

271   (Ott et al., 2015, Tang, Zhang et al., 2020), had the highest scores (Fig. 4a).

272   Interestingly, SAM50 and Mic60 showed a higher interaction score in EGFP-

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273   ORP5ΔPH immunoprecipitates, as compared to EGFP-ORP5. WB analysis using anti-

274   actin as loading control showed that EGFP-ORP5ΔPH overexpression did not alter the

275   amount of SAM50 and Mic60 proteins as compared to the overexpression of neither

276   EGFP-ORP5 nor EGFP alone (Fig. S4d), indicating that the higher interaction scores

277   of these proteins in the EGFP-ORP5ΔPH immunoprecipitates were not due to their

278   increased levels. Of note, metaxin-2 was also detected in the MS of

279   immunoprecipitated EGFP-ORP5 and EGFP-ORP5ΔPH, although its score was lower

280   than SAM50 and Mic60 and in the EGFP-ORP5 was below the assigned threshold (50)

281   (Fig. 4a).

282   To confirm ORP5/8 interaction with SAM50 and Mic60, GFP-pull down

283   experiments from HeLa cells expressing EGFP-ORP5, EGFP-ORP8 or EGFP alone

284   were carried out (Fig. 4b). Consistent with the MS data, endogenous SAM50 and Mic60

285   were recovered with both EGFP-ORP5 and EGFP-ORP8 but not with EGFP alone,

286   confirming specific biochemical co-purification of ORP5 and ORP8 with SAM50 and

287   Mic60.

288   Next, to determine the domains involved in the interaction of ORP5/8 with the

289   MIB/MICOS complex, GFP-pull down experiments were carried out from cells

290   expressing EGFP-tagged ORP5 (EGFP-ORP5ΔPH) or ORP8 (EGFP-ORP8ΔPH) PH

291   domain deleted constructs (Fig. S4a-b), and compared to the full-length proteins

292   (EGFP-ORP5 and EGFP-ORP8) or to the EGFP alone. In accord with the MS data,

293   the deletion of the PH domain increased ORP5 and ORP8 interaction with SAM50, as

294   compared to the full-length proteins (Fig. S4b). Confocal analysis of ORP5 and SAM50

295   localization in cells expressing EGFP-ORP5 or EGFP-ORP5ΔPH and stained with an

296   anti-SAM50 antibody confirmed the stronger enrichment of the ΔPH ORP5 construct

297   at ER elements in contact with the SAM50-labeled mitochondria as compared to the

298   full-length ORP5 (Fig. S4c).

299   As the PH domain is not required for the interaction with SAM50, we further

300   investigated the role of the other domains of ORP5 in such interaction by

301   immunoprecipitating ORP5 deletion mutants for the ORD or the TM domains (EGFP-

302   ORP5ΔORD, EGFP-ORP5ΔTM) (Fig. S4a-b). While the deletion of the ORD domain

303   did not affect the interaction between ORP5 and SAM50, the deletion of the TM domain

304   decreased the amount of SAM50 co-immunoprecipitated with ORP5, indicating that

305   ORP5 should be properly anchored to the ER to localize at ER-mitochondria contact

306   sites to interact with SAM50 (Fig. S4b).

307   To confirm the interaction between ORP5/8 and SAM50 at endogenous level we

308   took advantage of the available antibodies against ORP8 and SAM50 from different

309   species and analyzed their interaction using PLA (duolink) by confocal imaging in HeLa

310   cells. PLA signals corresponding to ORP8-SAM50 endogenous interaction were

311   detected at ER-mitochondria contact sites in control cells (Fig. 4c). Interestingly, PLA

312   signals were also detected for ORP8-metaxin 2, although the number of spots was

313   significantly minor (Fig. 4c-d), correlating to the lower MS score of metaxin 2 (Fig. 4a).

314   Then, to further verify the specificity of ORP8-SAM50 PLA we analyzed their

315   interaction in cells where SAM50 was downregulated by RNAi (siSAM50) (4e-f). A

316   decrease of about 40% of ORP8-SAM50 PLA was found in siSAM50 cells (Fig. 4f-g),

317   in accord with the decrease of the levels of SAM50 protein of about 40-50% assessed

318   by WB (Fig. 4e), and validating the specificity of the ORP8-SAM50 interaction.

319   Similarly, the KD of SAM50 decreased also the PLA interaction between ORP8 (and

320   ORP5) and metaxin 2 (Fig. S5a-d). To further investigate if this effect was specific for

321   protein components of the MIB complex we performed PLA interaction assays of ORP8

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322   (and ORP5) with another outer mitochondrial membrane protein TOM20, that is not a

323   bona-fide component of this complex. Interestingly, TOM20 also interacted with ORP8

324   and, to a minor extent with ORP5, but these interactions were not decreased by the

325   KD of SAM50 (Fig. 4h-i, S5e-f). Also, these effects were not due to decreased levels

326   of ORP5, ORP8, metaxin 2 and TOM20 proteins, as assessed by WB (Fig. 4e).

327   Together, our data reveal the specificity of the effects of SAM50 KD on ORP5/8

328   reciprocal interaction and with components of the MIB complex.

329   To investigate a possible role of SAM50 and Mic60 in regulating the levels of ORP5/8

330   at MAMs we analyzed their endogenous interaction by PLA and confocal microscopy

331   in HeLa cells where either SAM50 or Mic60 were knocked-down by siRNA.

332   Interestingly, a significant decrease (of about 40%) in ORP5/8 interaction in close

333   proximity to mitochondria (corresponding to MAM) was found in both SAM50 and

334   Mic60 KD cells, as compared to control cells (Fig. 5a-b), indicating a synergistic effect

335   of SAM50/Mic60 on the ORP5-ORP8 interaction at MAM. Further localization analysis

336   of endogenous ORP5 and ORP8 in siSAM50 cells confirmed decreased levels of these

337   proteins at MAM (Fig. 5 c-f).

338   To verify the possibility of an indirect effect of SAM50 or Mic60 silencing on the

339   morphology and abundance of MAMs we carried out an ultrastructural analysis by

340   conventional EM and HRP-KDEL EM (carrying a horseradish peroxidase (HRP) tagged

341   with an ER retention motif to stain the ER) in Mic60 or SAM50 silenced cells.

342   Morphological analysis by conventional EM showed that transient KD of SAM50 or

343   Mic60 induce formation of multilamellar cristae, almost devoid of CJ (Fig. S6a-b),

344   complementing previous observations by other groups through stable disruption of the

345   MICOS/MIB functions (Ding, Wu et al., 2015, Ott et al., 2015). However, in both SAM50

346   and Mic60 KD cells ER-mitochondria contact sites were still present and their

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347   morphology not altered (Fig. 5g, S6a). Quantitative morphological analysis by HRP-

348   KDEL EM in control and SAM50 or Mic60 silenced cells confirmed that the abundance

349   of ER-mitochondria contact sites was not altered by SAM50 or Mic60 KD (Fig. 5h),

350   indicating that the effects on ORP5/8 interaction at MAMs were not indirect due to a

351   global rearrangement of the ER-mitochondria contact sites.

352   Overall our data reveal a novel interaction between ORP5/8 and the MIB/MICOS

353   complex at ER subdomains associated with intra-mitochondrial membrane contacts

354   facing   CJ,   and   a   direct   role   of   the   MIB/MICOS   complex   in   ORP5/8

355   targeting/interactions at MAMs.

356

357   **ORP5/8 and the MIB/MICOS complex regulate PS-to-PE conversion at the ER-**

358   **mitochondria interface**

359   The role of ORP5 and ORP8 in lipid transport at ER-mitochondria contacts still

360   remains to be established. We tested whether ORP5 and ORP8 could mediate PS

361   transport at the ER-mitochondria interface by measuring levels of mitochondrial PE in

362   the mitochondrial fraction isolated from HeLa cells (as in (Galmes et al., 2016)) where

363   ORP5 or ORP8 were transiently silenced by RNAi. As the ER-derived PS is the major

364   precursor for mitochondrial PE, if ORP5 and ORP8 mediate non-vesicular transport of

365   PS from the ER to the mitochondria, their absence should lead to a reduction of

366   mitochondrial PE. We chose to use a transient KD as it overcomes the limits and/or

367   compensatory effects on lipid transport/biosynthetic pathways that other stable

368   approaches could induce. The purity of mitochondria and of the other subcellular

369   fractions was verified in control, ORP5 and ORP8 KD conditions by WB by probing the

370   samples for cytochrome c as mitochondrial marker and IP3R-3 as a MAM-enriched

371   marker (Fig. 6a). All markers were highly enriched in their respective fractions and

372 were absent in the others, and, as previously shown (Galmes et al., 2016), ORP5 and

373 ORP8 were enriched in the MAM fraction and absent in the mitochondria fraction of

374 control cells (Fig. 6a). On the contrary, they were strongly suppressed in ORP5 and

375 ORP8 KD cell lysates and in the respective MAM fractions (Fig. 6a). MS- lipidomic

376 analysis revealed a specific reduction of PE levels in mitochondria isolated from ORP5

377 and ORP8 KD cells, of 34% and 20%, respectively, as compared to control cells (Fig.

378 6b), while PE levels from total cells were unchanged. Additionally, the mitochondrial

379 levels of two other phospholipids (PI and PC) were not perturbed by ORP5/8 depletion

380 (Fig. S8a). Additionally, the decrease in PE could be due to a decrease in the protein

381 levels of the PS-decarboxylase (PISD) or the PS-Synthase 1 (PSS1) enzymes

382 mediating PS-to-PE conversion on mitochondria or PS synthesis in the ER,

383 respectively. To exclude this possibility, we analyzed the protein levels of PISD and

384 PSS1 by WB in ORP5, ORP8, ORP5+ORP8 KD cells and compared them to control

385 cells (Fig. S8b). We found no significant difference but rather a slight increase in the

386 enzymes upon ORP5 and/or ORP8 KDs. These data suggest that ORP5 and ORP8

387 could transfer PS at ER-mitochondria contact sites.

388   The ORD domain of ORP8 (ORD8) has been shown to transfer PS between liposome

389 membranes in counter-transport with PI4P or PIP_2 *in vitro* (Chung et al. 2015; Ghai et al,

390 2017). However, the lipid transfer activity of ORP5 ORD domain (ORD5) has not been

391 studied so far. Also, the ability of ORD5 and ORD8 to transfer PS independently of a

392 gradient of PI4P or PIP_2 or other phospholipids than PS has never been addressed. Thus,

393 we purified the recombinant ORD5 and ORD8 from bacteria (Fig. S7a) and compared

394 their ability to transport fluorescent phospholipids (TopFluor-PS, -PC or -PE) from donor

395 to acceptor liposomes *in vitro*. Donor liposomes containing fluorescent phospholipids and

396 biotinylated lipids were first immobilized on streptavidin beads and then mixed with

16

397 acceptor liposomes in the presence or absence of ORP5/8 ORD domains (Fig. S7b). After

398 1 hour at 37°C, acceptor liposomes were recovered from the supernatant and their

399 fluorescence was measured (Fig. S7b). Our results show that both ORD5 and ORD8

400 transfer PS, but not PC and PE, from donor to acceptor liposomes (Fig. S7c). They also

401 reveal that ORD5 and ORD8 share equivalent ability to transfer PS *in vitro*. To confirm

402 that fluorescent lipids were indeed transferred to the acceptor liposomes, a fraction of the

403 reaction supernatant was floated on a Nycodenz density gradient by ultracentrifugation

404 and the fluorescence in the top fraction of the gradient (containing floated acceptor

405 liposomes) was measured (Fig. S7d). Fluorescence of TopFluor-PS in the acceptor

406 liposomes was maintained after their floatation, confirming its effective transfer between

407 liposomes *in vitro*.

408   In subsequent experiments, we measured the levels of mitochondrial PE newly

409 synthesized from the ER-derived PS by using a radiometric PS-to-PE conversion

410 assay *in situ* (Shiao, Lupo et al., 1995) in silenced or control HeLa cells (Fig. 6c). This

411 assay allows the monitoring of PS transfer from the ER to mitochondria by measuring

412 the levels of radioactive PS and PS-derived PE by thin layer chromatography (TLC)

413 after incorporation of radioactive L-[^{3}H(G)]-serine into the cells. A significant decrease

414 in the levels of newly synthetized PE was found in ORP5 and in ORP5+ORP8 KD cells

415 (Fig. 6c). The decrease was stronger in ORP5+ORP8 KD cells, indicating a

416 cooperative effect of ORP5 and ORP8 in this process. A slight, not statistically

417 significant decrease was also found in ORP8 KD cells, suggesting a major role of

418 ORP5 compared to ORP8 in PS transfer at ER-mitochondria contact sites *in situ*. As

419 [^{3}H]-serine radioactivity could be incorporated to PE also *via* an alternative pathway

420 involving sphingosine (Hanada, Nishijima et al., 1992), we next sought to address the

421 contribution of this pathway to PE labeling, by repeating the experiments in control and

17

422   ORP5 KD cells in the presence throughout pulse and chase of [3H]-serine of β-chloro-

423   L-alanine, an inhibitor of serine palmitoyltransferase (Chen, Born et al., 1993).

424   Although a decrease in newly synthesized PE was observed in siCtrl cells upon

425   addition of β-chloro-L-alanine, PS-to-PE conversion was significantly reduced in β-

426   chloro-L-alanine treated and untreated ORP5 knockdown cells (~17% and ~23%

427   respectively), as compared to control (Fig. 6d). We then verified that the fraction of PE

428   decreased upon ORP5 KD was indeed derived from conversion of PS by PISD by

429   measuring PS-to-PE conversion in the presence of hydroxylamine, an inhibitor of PISD

430   activity (Fig. 6e). Treatment with 5 mM hydroxylamine robustly inhibited the PS to PE

431   conversion in both control and ORP5 KD cells, the level of residual conversion being

432   similar in both of these cells, further confirming that the reduction in mitochondrial PE

433   induced by depletion of ORP5 is essentially due to the decrease in PS transfer from

434   the ER to the mitochondria (Fig. S8e). The β-chloro-L-alanine and hydroxylamine data

435   also suggest that at least 80% of the serine labeling of PE occurs via the

436   phosphatidylserine decarboxylase reaction in HeLa cells, in accord with a previous work

437   in another cell type, the Baby Hamster Kidney fibroblasts (BHK cells), showing that only

438   certain minor PE species are labeled from the sphingosine-PE pathway (Heikinheimo

439   & Somerharju, 1998).

440   To test whether the effects on PS transport at ER-mitochondria contacts were

441   specific for ORP5 and ORP8 loss of function or simply due to a decrease of ER-

442   mitochondria contacts induced by their KD, we quantified the abundance of ER-

443   mitochondria contact sites by EM in control and ORP5, ORP8, and ORP5+ORP8 KD

444   cells. To facilitate the visualization of the ER we transfected the cells with a HRP-KDEL

445   construct that stains the ER with a dark signal. Our quantifications revealed that ORP5,

446   ORP8 or ORP5+ORP8 KDs did not affect the extent of ER-mitochondria contacts (Fig.

18

447   6f-g). The total mitochondrial mass (quantified as mitochondrial surface/cell) was also

448   unchanged (Fig. S8c). These results indicate that ORP5 and ORP8 act as LTPs rather

449   than tethers (Fig. 7, S8e). Even a modest reduction (22-27%) of mitochondrial PE

450   levels in mammalian cells has been shown to profoundly alter the morphology of

451   mitochondrial cristae as well as mitochondria functions (Tasseva, Bai et al., 2013).

452   Accordingly, 52% of mitochondria in ORP5+ORP8 double-KD cells display aberrant

453   cristae morphology versus 9% in control cells (Fig. 6f, S8d). These defects in cristae

454   morphology were also observed by conventional EM (Fig. S6a) and were similar to

455   those previously shown in the case of ORP5 and ORP8 individual KD (Galmes et al.,

456   2016). Interestingly, the % of mitochondria with altered morphology in ORP5+ORP8

457   double-KD cells was higher as compared to ORP8 KD (Galmes et al., 2016), reflecting

458   the stronger effect of ORP5+ORP8 double-KD on PS transport at ER-mitochondria

459   contact sites (Fig. 6c, S8d).

460   Decreased levels of PE strongly affect the organization of the mitochondrial

461   respiratory supercomplexes (Tasseva et al., 2013). We had previously shown that

462   ORP5 KD induces a reduction in the basal mitochondrial oxygen consumption rate

463   (OCR_{BAS}), indicative of reduced respiratory activity (Galmes et al., 2016). However, it

464   remains still questioned whether ORP8 could also reduce OCR_{BAS} and if this reduction

465   could be exacerbated under metabolic stress conditions. Thus, we monitored

466   mitochondrial OCR in control, in ORP5 and in ORP8 KD cells in basal and in stress

467   conditions (Fig. S9). ORP5 KD induced a significant reduction in both OCR_{BAS} (~37%)

468   and OCR upon FCCP treatment (OCR_{FCCP}) (~36%). Interestingly, also ORP8 KD

469   induced a significant decrease in OCR_{BAS} (~31%) and OCR_{FCCP} (~29%), although the

470   decreases were less prominent than upon ORP5 KD. These data uncover a novel role

471   of ORP5 and ORP8 in preserving mitochondrial respiratory activity in basal and in

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472   stress conditions, and a major impact of ORP5 in this process, in accord with its major

473   role in PS transfer at ER-mitochondria contact sites.

474   To test whether the interaction of ORP5 with the MIB complex could facilitate

475   the non-vesicular transfer of PS from the ER to the mitochondrial membranes (and

476   consequently synthesis of mitochondrial PE) we performed radiometric PS-to-PE

477   conversion assay in cells depleted of ORP5, SAM50 or Mic60 alone or in

478   combination by RNAi. Robust KD of ORP5, SAM50 or Mic60 was confirmed by

479   western blotting after 48 hours (Fig. 6h). Analysis of PS-derived newly synthetized

480   PE revealed a significant decrease in PE in ORP5 and Mic60 KD cells (Fig. 6i).

481   Moreover, the double-KD of ORP5 and Mic60 had an additive effect, supporting a

482   cooperation of these two proteins in the regulation of PE synthesis. However, the

483   levels of PE were not changed in SAM50 KD cells as compared to control. This

484   could be explained by the fact that other subunits of the MIB complex might

485   compensate for its depletion. Indeed, protein levels of Mic60 and metaxin 2 were

486   strongly increased upon SAM50 KD (Fig. 4e, 6h). Interestingly, the double-silencing

487   of SAM50 and either ORP5 or Mic60 had a significant impact on PE synthesis that

488   was even stronger as compared to the individual KDs (Fig. 6i).

489   EM analysis of mitochondria morphology in all KD conditions revealed that

490   downregulation of ORP5, SAM50 and Mic60, individually or in combination, induces

491   formation of multilamellar cristae, almost devoid of CJ (Fig. S6a-b). However, in all KD

492   conditions ER-mitochondria contact sites were still present and their morphology was

493   not altered (Fig. 5g-h, Fig S6a), indicating that the effects on PS-derived PE synthesis

494   were specifically due to ORP5, Mic60 or SAM50 loss of function effects on PS transport

495   at ER-mitochondria contacts or on the maintenance of intra-mitochondrial membrane

496   bridges, respectively.

20

497   Overall our results reveal that ORP5/8 cooperate with the MIB/MICOS complex to

498   regulate the transfer of PS from the ER to the mitochondrial membranes necessary for

499   synthesis of mitochondrial PE and consequently for maintaining mitochondrial cristae

500   morphology and respiration (Fig. 7).

501

502   **DISCUSSION**

503   In this study, by using a combination of biochemical and imaging approaches, we

504   uncover for the first time the endogenous localization of ORP5 and ORP8, revealing that

505   they are mainly localized at ER-mitochondria contact sites and providing novel evidence

506   for a physiological relevance of the ORP5/8 complex at MAMs (Fig. 1-2 and Fig. S1-3).

507   So far, ORP5 and ORP8 localization have been only studied in conditions where one of

508   these two partner proteins were expressed in high excess as compared to the other one.

509   Previous studies, including one from our group, have shown that overexpression of ORP5

510   induces an increase of ER-PM contacts where the protein also localizes (Chung et al.,

511   2015, Galmes et al., 2016). Consequently, several following studies have addressed the

512   role of ORP5 and ORP8 at ER-PM contacts, overlooking their function at MAM. However,

513   the increase in cortical ER observed upon ORP5 overexpression does not reflect the

514   physiological abundance of ER-PM contacts, as the cortical ER in non-specialized cells

515   generally covers not more than 5% of the plasma membrane surface. Indeed, we have

516   found that the increased localization of ORP5 to cortical ER when over-expressed alone

517   does not reflect its endogenous localization when it is in complex with ORP8, which is

518   instead enriched at MAMs. Our findings have important implications for a better

519   understanding of the physiological localization and function of ORP proteins, but also of

520   other proteins that assemble in multimeric complexes at ER-mediated membrane contact

521   sites, such as the E-Syts (Giordano, Saheki et al., 2013).

522   Our study reveals that ORP5/8 physically interact with SAM50 and Mic60, two key

523   components of the MIB/MICOS complex that anchor the IMM to the OMM at the level of

524   CJ (Huynen et al., 2016, Ott et al., 2015, Wollweber et al., 2017). The biochemical

525   interaction between ORP5 and SAM50/ Mic60 uncovers the existence of a novel physical

526   link between ER-mitochondria contact sites involved in lipid transport and intra-

527   mitochondrial membrane contacts. ORP5 localization by IEM at ER-mitochondria contact

528   sites near the CJ, where Mic60 and MICOS complex also reside, further confirms the

529   existence of such tripartite membrane contact site structure. Moreover, KD of SAM50 and

530   Mic60 does not affect ER-mitochondria contact sites, but specifically perturbs ORP5/8

531   interactions with themselves and with metaxin 2, another component of the MIB complex

532   at MAMs.

533   Importantly, here we describe a new function of ORP5 and ORP8 in the maintenance

534   of mitochondrial levels of PE, an essential phospholipid of mitochondria, providing the first

535   evidence of mammalian LTPs directly mediating non-vesicular transfer of PS (lipid

536   precursor of mitochondrial PE) from the MAMs to the mitochondria at ER-mitochondria

537   contact sites.

538   Other LTPs have been recently identified at ER-mitochondria contact sites in

539   mammalian cells. Hirabayashi et al. recently showed that the SMP-containing protein

540   PDZD8 is involved in ER-mitochondria tethering and in the regulation of Ca^{2+} dynamics

541   in mammalian neurons (Hirabayashi et al., 2017). Although PDZD8 is a structural and

542   functional paralogous of the Mmm1 subunit of the ERMES complex (Wideman et al.,

543   2018), its function in lipid transport at ER-mitochondria contact sites remains unclear.

544   Recently, the mammalian LTP VPS13A has been shown to localize to contact sites

545   including ER-mitochondria contacts (Kumar et al., 2018). VPS13A contains a lipid-

546   binding domain (VPS13α) that has the ability to harbor multiple phospholipids at once

22

547   and transfer them between liposomes *in vitro*. However, its role in lipid transfer at ER-

548   mitochondria membrane contact sites *in situ* has not yet been established. Differently

549   from the SMP and VPS13α domains that can simultaneously host multiple

550   phospholipids, the ORD domain of Osh/ORPs forms a cavity that can host only one

551   lipid at a time (Maeda, Anand et al., 2013, Wang, Ma et al., 2019).

552   An important finding of our work is that ORP5 and ORP8 KD do not affect the

553   extent of ER-mitochondria contact sites, revealing that the main function of ORP5/8 at

554   MAMs is lipid transfer and not membrane tethering. This is a unique feature among

555   the LTPs that have been identified so far at MAMs. For instance, KD of PDZD8 and

556   VPS13A result in a decrease of ER-mitochondria contact sites (Hirabayashi et al.,

557   2017, Kornmann, Currie et al., 2009, Kumar et al., 2018), making it difficult to dissect

558   the lipid transfer activity from the tethering function of these LTPs. Thus, ORP5/8

559   represent a so far a unique example of LTPs that specifically mediate lipid transport at

560   ER-mitochondria contact sites, independently of membrane tethering, and that can be

561   used as unique tools to specifically study lipid transport at MAMs.

562   ORP5 and ORP8 have been previously shown to counter-exchange PS with the PM

563   phosphoinositides PI4P and PIP_2 at ER-PM contact sites in HeLa cells (Chung et al.,

564   2015, Ghai, Du et al., 2017). However, PI4P and PIP_2 are not present on the

565   mitochondrial membranes, while PE is highly abundant in these membranes, in

566   addition to being an essential lipid of all biological membranes. Our *in vitro* data show

567   that the ORD domains of ORP5 and ORP8 transport PS, but not other phospholipids such

568   as PE and PC, indicating a specific role of ORP5/8 in PS transport and excluding the

569   possibility that ORP5/8 might also participate in the transport of a fraction of PE back

570   to the ER. It is possible that ORP5/8 cooperate with other LTPs, such as VPS13A, for the

571   exchange of other lipids (including PE) at ER-mitochondria contact sites. Importantly,

23

572   we have confirmed the role of ORP5/8 in PS transfer by measuring a specific decrease

573   of PS-derived mitochondrial PE in ORP5 depleted HeLa cells *in situ* (and even more

574   upon ORP5+8 silencing) as well as a reduction of total PE in mitochondria isolated

575   from these cells (Fig. 6a-e, S8a-b). Accordingly, ORP5/8 KD affects cristae morphology

576   and the respiratory function of mitochondria (Galmes et al., 2016)(Fig. 6f-g, S6, S8d),

577   all phenotypes that are expected in the case of even a mild decrease in mitochondrial

578   PE (Joshi et al., 2012, Steenbergen et al., 2005);(Tasseva et al., 2013). Our data also

579   suggest that the gradient of PS at ER-mitochondria contacts is sufficient to trigger the

580   ORP5/8-mediated transport of PS from the MAMs, where it is highly enriched, to the

581   mitochondria membranes, where it is rapidly converted into PE and is therefore present

582   at a very low concentration. Our findings have important implications in the general field

583   of LTPs, as they suggest that the same LTP can use different means to transfer lipids

584   depending on the local gradients present at the specific membrane contact sites where

585   it is localized.

586   Importantly, we also show that the *de-novo* synthesis of mitochondrial PE requires

587   both ORP5/8 at ER-mitochondria contact sites and the MIB/MICOS complex at intra-

588   mitochondrial OMM-IMM contact sites (Fig. 6i). Interestingly, recent evidence in yeast

589   suggests that, in addition to the classical PE synthesis at the IMM by the IMM-localized

590   PS-decarboxylase PISD, PE can also be synthesized *in trans* on the OMM (Aaltonen,

591   Friedman et al., 2016). Thus, it is possible that this alternative pathway, which requires

592   MIB/MICOS tethering function to bring the mitochondrial intermembrane domain of

593   PISD close to the OMM for synthesis of PE, is conserved also in mammalian cells. The

594   cooperation of ORP5 (through its lipid transfer activity at ER-mitochondria contact sites)

595   with SAM50 and Mic60 (through their OMM-IMM tethering function) could facilitate the

596   movement of PS from the ER to the IMM where it is converted to PE, through the

597   classical PE synthesis pathway or through PISD function *in trans* on the OMM. Taken

598   together these findings provide the first evidence of a physical and functional link

599   between ER-mitochondria contacts and intra-mitochondrial membrane contacts

600   maintained by the MIB/MICOS complexes, to facilitate transport of PS from the ER to

601   the mitochondria and PE synthesis on the mitochondrial membranes (Fig. 7).

602   In conclusion, our data reveal that: 1) ORP5/8 form a protein complex that is

603   endogenously enriched at ER-mitochondria contacts; 2) ORP5/8 constitute a molecular

604   machinery mediating PS transfer at ER-mitochondria contact sites but not ER-

605   mitochondria tethering; 3) ER-mitochondria contacts where ORP5/8 localize are

606   physically associated with intra-mitochondrial contacts, maintained by the MIB/MICOS

607   complex, to facilitate the transport of PS from the ER to mitochondria membranes.

608   Overall our study provides a first molecular clue on how lipids are transported at ER-

609   mitochondria contact sites and novel functional insight into the complex interplay of the

610   ER with the mitochondria and the intra-mitochondrial membrane contacts and associated

611   machinery.

612

613   **Figure Legends**

614   **Figure 1. Endogenous and co-overexpressed ORP5 and ORP8 co-localize at**

615   **ER-mitochondria contact sites.** (a) Confocal images of Ctrl, ORP5 and ORP8

616   knockdown HeLa cells were immunostained using anti-ORP5 or ORP8 antibodies

617   (green), and treated with MitoTracker to label mitochondria (red) and DAPI to stain the

618   nuclei (blue). Images are presented as individual layers. Insets show magnifications of the

619   boxed regions. Note the close association of endogenous ORP5 and ORP8 to

620   mitochondria. Scale bar, 10 μm. (b) WB analysis showing ORP5, ORP8 and GAPDH

621   levels in protein lysates from Ctrl, ORP5 and ORP8 knockdown HeLa cells. (c)

622   Quantification of ORP5 and ORP8 fluorescent intensity in Ctrl, ORP5 and ORP8

623   knockdown cells. Mean of fluorescent intensities in arbitrary units (a.u. x10^5). Error bars

624   denote ± standard error of the mean (SEM). Number of cells given above bars. Statistical

625   analysis: P values were determined by unpaired student's t-test, *P<0.05, ***P<0.001. (d)

626   Distribution of ORP5 and ORP8 IF staining (spots) in relation to their distance (in µm) to

627   mitochondria indicating that part of the endogenous ORP5 and ORP8 in the cell is

628   detected in close proximity to mitochondria (<0.5 µm). (e) Confocal images of a HeLa cell

629   immunostained using anti-ORP5 (green) or ORP8 (red) antibodies and MitoTracker

630   (blue). Images are presented as individual layers. Insets show magnifications of the boxed

631   regions. Scale bar, 10 µm. (f) Quantificiation of the co-localization (Pearson's factor) of

632   ORP5-ORP8 in endogenous (ORP5-8 end) and co-overexpression (ORP5-8 over)

633   conditions. Bars indicate mean values ± SEM. Number of cell analyzed: ORP5-8 end

634   (n=15), ORP5-8 over (n=14). (g) Confocal micrograph of a region of HeLa cell (zoomed

635   from Fig EV1a) transfected with EGFP-ORP5 (green), EGFP-ORP8 (green) or EGFP-

636   ORP8 (green) + HA-ORP5 (anti-HA, red), and with Mito-BFP (blue). (h) Quantifications of

637   the association to mitochondria (Pearson's factor) of the indicated EGFP-tagged

638   constructs. Bars indicate mean values ± SEM of three independent experiments with 10

639   cells for sample analyzed in each experiment (n=30). Statistical analysis: unpaired

640   Student's t-test comparing EGFP-ORP5 (ORP5) or EGFP-ORP8 (ORP8) to EGFP-

641   Sec22b (SEC22b) and HA-ORP5 (+EGFP-ORP8) or EGFP-ORP8 (+HA-ORP5) to

642   EGFP-ORP5 or EGFP-ORP8, respectively. *P<0.05, **P<0.01.

643

644   **Figure 2. The main localization of the endogenous ORP5-8 complex is ER-**

645   **mitochondria contact sites.** (a) Representative confocal images of ORP5-ORP8

646   interaction in HeLa cells detected by Duolink PLA (green spots) in endogenous (ORP5-

647   ORP8 Endo) and overexpressing (HA-ORP5 + 3xFL-ORP8) conditions, and their

648   respective 3D representation by Imaris. Images are presented as maximum projection of

649   all layers. Insets show magnifications of the boxed regions. Scale bar, 10 µm. (b)

650   Quantification of the number of ORP5-ORP8 PLA interactions in Control (siCtrl, n=39

651   cells), ORP5 and ORP8 knockdown (siORP5+8, n=38 cells), and in overexpression of

652   ORP5 and ORP8 (Ovrexp ORP5+8, n=35 cells), showing that the downregulation or the

653   upregulation of both ORP5 and ORP8, respectively, reduces and increases the number

654   of interaction stablished between these two proteins. Statistical analysis: P values were

655   determined by unpaired student's t-test, ***P<0,001. (c) Quantification of ORP5-ORP8

656   PLA interaction localized to ER-Mitochondria contact sites in control (Endo, n= 33) and

657   HeLa cells overexpressing (HA-ORP5 + 3xFL-ORP8, n=27 cells) showing that about 50%

658   of ORP5-ORP8 interactions occurs at MAM. (d) Representative confocal images of

659   ORP5-ORP8 PLA interaction (green spots) detected in HeLa cells overexpressing

660   PHPLCd-RFP, Mito-BFP (ORP5-ORP8 Endo), or in HeLa cells overexpressing PHPLCd-

661   RFP, Mito-BFP, ORP5 and ORP8 (HA-ORP5 + 3xFL-ORP8). Images are presented as

662   individual layers. Scale bar, 10 µm. (e) Quantification of ORP5-ORP8 PLA signal localized

663   to ER-plasma membrane contact sites indicate that about 5% of the total ORP5-ORP8

664   interactions occurs to these subdomains of the ER membranes, in both   control

665   (Endogenous, n=6 cells) and HeLa cells overexpressing ORP5 and ORP8

666   (Overexpressed, n=14 cells). (f) Box plot of ORP5-ORP8 endogenous PLA spots distance

667   (in µm) to mitochondria and plasma membrane (box around median value, whiskers 10%-

668   90%) evidencing that the majority of ORP5-ORP8 interactions were detected in a close

669   proximity to mitochondria (<0.38 µm) and distant from the plasma membrane (≥0.38 µm).

670

27

671   **Figure 3. ORP5 localizes at ER-mitochondria contact sites near cristae**

672   **junctions (CJ).** (a) Electron micrographs of ultrathin cryosections of HeLa cells

673   transfected with HA-ORP5 or Mic60-EGFP and immunogold stained with anti-HA or anti-

674   GFP (10 or 15 nm gold), showing ORP5 localization at ER-mitochondria contacts in close

675   proximity to CJ (arrow) and the localization of the MICOS complex (Mic60) at CJ (arrows).

676   Scale bar 250 nm. (b) Quantification of the proximity of HA-ORP5 and EGFP-Sec61b gold

677   particles to the CJ. Results are presented as the percentage of ORP5 or Sec61b gold

678   particles at specific ranges of distance (in nm) from CJ. 150 gold particles were counted

679   on randomly selected cell profiles in each sample. (c) Electron micrographs of ultrathin

680   cryosections of HeLa cells transfected with EGFP-ORP5 or GFP-Sec61b and

681   immunogold labeled with anti-GFP (15 nm gold) and anti-PDI (10nm gold). Note ORP5

682   localization at ER-mitochondria contacts near CJ (arrow) and Sec61b localization to ER

683   membranes not in contact with the mitochondria membranes (arrowheads). Scale bar 250

684   nm. (d) Electron micrographs of ultrathin cryosections of HeLa cells transfected with

685   EGFP-ORP8 or EGFP-ORP5 and PTPIP51-HA and immunogold labeled with anti-GFP

686   (15 nm gold) and anti-HA (10nm gold). Note ORP8 and ORP5 specific localization at ER-

687   mitochondria contacts near CJ (black arrowheads) and PTPIP51 localization to all the the

688   mitochondria surface including CJ (red arrows). Scale bars 250 nm. (e) Quantification of

689   PTPIP51-HA, EGFP-ORP8 and EGFP-ORP5 proximities to CJ. Results are presented as

690   the % of gold particles within 0-50nm distance from CJ±SEM. 286 gold particles for

691   PTPIP51 and 150 gold particles for ORP5 and ORP8 were counted on randomly selected

692   cell profiles in each sample. Statistical analysis: unpaired student's t-test, **P<0.01.

693

694   **Figure 4. ORP5 and ORP8 interact with the MIB/MICOS complex at ER-**

695   **mitochondria contacts.** (a) Identification of mitochondrial proteins associated to

696   mitochondrial outer or inner membranes (OMM, IMM) that interact with EGFP-tagged

697   ORP5 constructs by mass spectrometry. Note the presence of some proteins of the MIB

698   complex: Mic60, SAM50 and metaxin2 and of their interacting partner RHOT2. Interaction

699   scores (Mascot scores) of Mic60, SAM50 and metaxin2 with the EGFP-ORP5ΔPH

700   construct are stronger than with EGFP-ORP5. (b) Western blot analysis of ORP5, ORP8,

701   SAM50 and Mic60 in immuno-precipitated samples obtained from lysates of HeLa cells

702   transfected with EGFP-ORP5, EGFP-ORP8 or EGFP alone. ORP5 and ORP8 were

703   detected using antibodies against GFP. (c) Representative confocal images showing

704   endogenous ORP8-SAM50 and ORP8-metaxin2 PLA interactions (green), mitochondrial

705   network (MitoTracker, red) and nuclei (DAPI, blue) in HeLa cells. Images are displayed as

706   maximum projection of all layers. Scale bar, 10 μm. (d) Quantitative analysis of

707   endogenous ORP8-SAM50 and ORP8-metaxin2 PLA interactions in HeLa cells. Data are

708   shown as mean values ±SEM of n= 36 cells, ORP8-SAM50; n= 39 cells, ORP8-Metaxin2.

709   Statistical analysis was performed using unpaired student's t-test, with ****, P<0.0001. (e)

710   Western blot analysis of ORP5, ORP8, SAM50, TOM20 and metaxin2 in protein lysates

711   obtained from HeLa cells treated with scrambled siRNA (siCtrl) and from Hela cells treated

712   with siRNA targeting SAM50 (siSAM50). (f) Confocal images of control (siCtrl) and SAM50

713   (siSAM50) knockdown HeLa cells showing endogenous interaction of ORP8-SAM50 by

714   Duolink PLA (green) at MAMs. Mitochondria are labeled by MitoTracker (red) and nuclei

715   by DAPI (blue). Images are presented as maximum projection of all layers. Insets show

716   magnifications of the boxed regions. Scale bar, 10 μm. (g) Quantification of endogenous

717   ORP8-SAM50 PLA signals in Control and SAM50 knockdown HeLa cells, showing the

718   reduction of about 50% of ORP8-SAM50 PLA in SAM50 knockdown cells as compared

719   to control. Bars indicate mean values ±SEM. Number of cell analysed: siCtrl (n=30),

720   siSAM50 (n=22). Statistical analysis: P values were determined by unpaired student's t-

29

721 test, **P<0.01. (h) Representative confocal images showing endogenous ORP8-TOM20

722 PLA interactions (green), mitochondria (MitoTracker, red) and nuclei (DAPI, blue) in

723 Control and SAM50 knockdown HeLa cells. Images are displayed as single layers. Scale

724 bar, 10 μm. (i) Quantitative analysis of endogenous ORP8-TOM20 PLA interactions in

725 HeLa cells. Data are shown as mean values ±SEM of n=49 cells, siCtrl; n= 49 cells,

726 siSAM50, n.s; not significant.

727

728 **Figure 5. SAM50 and Mic60 knockdowns induce a decrease in ORP5-8**

729 **interaction at MAMs but do not alter the abundance of ER-mitochondria contact**

730 **sites.** (a) Confocal images of a region of Ctrl (siCtrl), SAM50 (siSAM50) and Mic60

731 (siMic60) knockdown HeLa cells showing endogenous interaction of ORP5-8 by Duolink

732 PLA (green) near mitochondria (MitoTracker, red). Scale bar, 5 μm. (b) Quantification of

733 ORP5-8 PLA signals in Control, SAM50 and Mic60 knockdown HeLa cells, showing the

734 decrease of about 40% of ORP5-ORP8 PLA in SAM50 and Mic60 knockdown cells as

735 compared to control. Bars indicate mean values ±SEM. Number of cell analysed: siCtrl

736 (n=33), siSAM50 (n=19), siMic60 (n=24). Statistical analysis: unpaired sudent's t-test.

737 ***P<0.001. (c-d) Confocal images showing endogenous ORP5 (green) and ORP8

738 (green) localization in relation to mitochondria (MitoTracker, red) in Ctrl (siCtrl) and SAM50

739 (siSAM50) knockdown HeLa cells. Nuclei are stained with DAPI (blue). Images are

740 represented as single layers. Magnifications of wite boxed regions are shown for each

741 condition. Scale bar, 10 μm. (e-f) Quantitative analysis of ORP5 and ORP8 distribution in

742 relation to their distance to mitochondria in HeLa cells treated with scrambled siRNA

743 (siCtrl) and siRNA targeting SAM50 (siSAM50). Violin plots display overall frequency and

744 distribution of ORP5 and ORP8 labelling in relation to their distance to mitochondria.

745 Median values (wider dotted line) anti-ORP5; siCtrl= 0.61 μm, siSAM50= 1.04 μm; and

746   anti-ORP8: siCtrl= 0.48 µm, siSAM50= 0.84 µm. Interquartile range (narrow dotted lines)

747   anti-ORP5; siCtrl 25% and 75% percentile= 0.32 and 1.26 µm, siSAM50 25% and 75%

748   percentile= 0.48 and 1.97 µm. Anti-ORP8: siCtrl 25% and 75 % percentile= 0.23 and 1.01

749   µm, siSAM50 25% and 75% percentile= 0.34 µm and 1.7 µm. Column charts display the

750   % of ORP5 and ORP8 labelling that distance from mitochondria 0 µm, <0.38 µm and <0.5

751   µm. Data are expressed as mean values ±SEM. Number of cell analyzed: anti-ORP5:

752   siCtrl n=37, siSAM50 n= 40; anti-ORP8: siCtrl n=22, siSAM50 n= 29. Statistical analysis:

753   P values were determined by unpaired student's t-test, *** P<0.001. (g) Representative

754   electron micrographs of HeLa cells treated with Ctrl siRNAs or siRNAs against SAM50 or

755   Mic60 and transfected with HRP-KDEL. Red arrows indicate ER-mitochondria contact

756   sites. Scale bar, 500 nm. (h) Quantifications of the extent of ER-mitochondria contact sites

757   in Ctrl, Mic60 and SAM50 knockdown cells expressing HRP-KDEL. Data are shown as %

758   of the ER in contact with mitochondria (mitochondria occupancy) ±SEM, n = 30 for siCtrl,

759   n = 20 cell profiles for siMic60 and siSAM50 and 1000 mitochondria; n.s; not significant.

760

761   **Figure 6. ORP5/8 and the MIB/MICOS complex regulate levels of PS-derived**

762   **mitochondrial PE.** (a) Crude mitochondria, mitochondria, and MAM fractions were

763   purified from Ctrl, ORP5 and ORP8 siRNA-treated HeLa cells. Equal amounts of protein

764   from each fraction were loaded on a 4–20% gradient SDS–PAGE gel and immunoblotted

765   using anti-ORP5, anti-ORP8, anti-IP3R-3 (MAM protein), and anti-cytochrome c

766   (mitochondrial protein). Mito, mitochondria; MAM, mitochondria-associated ER

767   membrane. (b) Mass spectrometry (MS)-based quantification of the PE content (nmol/mg

768   protein) of mitochondria isolated from Ctrl, ORP5 or ORP8 knockdown cells and of Ctrl,

769   ORP5 or ORP8 knockdown intact cells. Data are shown as mean of three independent

770   replicates ±SEM. Statistical analysis: unpaired student's t-test, *P<0.05, **P<0.01. (c)

31

771   HeLa cells transfected with siCtrl, siORP5, siORP8 or siORP5+ORP8 RNAi oligos were

772   incubated with L-[^{3}H(G)]serine (30.9 Ci/mmol) for 18 hours. After extraction and separation

773   of lipids by TLC, PS and PE spots were scraped and analyzed for [^{3}H] radioactivity, as

774   described under "Methods". Each condition was analyzed in triplicate in each of three

775   independent biological replicates. Data are presented as mean of PE:PS ratio ±SEM.

776   Statistical analysis: unpaired student's t-test, *P<0.05, **P<0.01 compared to Ctrl. (d) Cells

777   transfected with siCtrl and siORP5 oligos were treated with 1 mM β-Chloro-L-alanine (CA,

778   β-Chl-L-ala, inhibitor of Ser-palmitoyltransferase) or untreated, then pulsed with 7 μCi/ml

779   of [3H(G)]serine for 1 hour and chased for 4 hours in serum-free DMEM + β-Chloro-L-

780   alanine, before analysis.   n.s. not significant, **P<0.01 compared to Ctrl. n.s. not

781   significant. (e) Similar experiments were performed in presence of 5 mM hydroxylamine

782   (HA, inhibitor of phosphatidylserine decarboxylase). **P<0.01, n.s. not significant. (f)

783   Electron micrographs of HRP-KDEL-expressing HeLa cells treated with Ctrl siRNAs

784   (siCtrl) or siRNAs against ORP5 and ORP8 (siORP5+siORP8). Red arrows indicate ER-

785   mitochondria contact sites. Scale bar, 500 nm. (g) Quantifications of the total extent of ER-

786   mitochondria contact sites in siCtrl, siORP5, siORP8 and siORP5+8 cells expressing

787   HRP-KDEL. Data are shown as % of the ER surface lenght in contact with mitochondria

788   ±SEM, n = 20 cell profiles and ±900 mitochondria; n.s; not significant. (h) Western analysis

789   showing ORP5, SAM50, Mic60 and Actin levels in protein lysates from HeLa cells treated

790   with siRNA against Ctrl, ORP5, Mic60 or SAM50. Arrow indicates the specific band for

791   Mic60. (i) Radiometric measurement of PS-to-PE conversion in the indicated siRNAs.

792   Data are presented as mean of PE:PS ratio ±SEM. Each condition was performed in

793   triplicate in each of the independent biological replicates (n = 5 for siCtrl and siORP5; n =

794   4 for siSAM50; n = 3 for the other siRNAs conditions). Statistical analysis: unpaired

795   student's t-test , *P<0.05, **P<0.01 compared to Ctrl.

32

796

797   **Figure 7. PS transport at ER-mitochondria contact site subdomains associated**

798 **to MIB/MICOS complex.** ORP5/8 mediate the transfer of PS from ER to mitochondria at

799 ER-mitochondria membrane contact sites. This transfer occurs at ER subdomains facing

800 the cristae junctions (CJ) where ORP5/8 interacts with SAM50 and Mic60, key proteins of

801 the MIB complex. This interaction facilitates the transfer of PS from ER to the

802 mitochondrial membranes at the level of CJ and PS conversion into PE, a phospholipid

803 that plays a critical role in cristae organization and mitochondrial function.

804

805   **Figure S1. Antibodies anti-ORP5 and anti ORP8 specifically bind ORP5 and**

806 **ORP8, respectively.** (a) Confocal images of a region of HeLa cell transfected with EGFP-

807 ORP5 (green) or EGFP-ORP8 (green) and immunostained using anti-ORP5 (red) or anti-

808 ORP8 (red) antibodies. Scale bar, 5 μm. (b) Quantifications of the colocalization

809 (Pearson's factor) of EGFP-ORP5 or EGFP-ORP8 with anti-ORP5 or anti-ORP8. Bars

810 indicate mean values ±SEM. Cells analyzed for sample: n=5. (c-e) Western blot analysis

811 using primary antibodies against ORP5 and ORP8 in protein samples obtained from HeLa

812 cells treated with scrambled siRNA (siCtrl) and from Hela cells treated with siRNA

813 targeting ORP5 (siORP5) or ORP8 (siORP8). Images show the full membranes which

814 were firstly incubated with anti-ORP5 or anti-ORP8, and then with anti-GAPDH. (e)

815 Analysis of ORP5 and ORP8 expression in siCtrl and siORP5 and siORP8, respectively,

816 by immunofluorescence and Western blot.

817

818   **Figure S2. Endogenous and co-overexpressed ORP5 and ORP8 co-localize at**

819 **ER-mitochondria contacts.** (a) Confocal micrographs of regions of HeLa cell transfected

33

820   with RFP-Sec22b (red) together with Mito-BFP (blue) and immunostained for anti-ORP5

821   (green) or anti-ORP8 (green) antibodies. Scale bar, 5 µm. (b) Confocal micrograph of a

822   HeLa cell transfected with EGFP-ORP5 (green), EGFP-ORP8 (green) or EGFP-ORP8

823   (green) + HA-ORP5 (anti-HA, red), together with Mito-BFP (blue). The boxed areas are

824   magnified in Fig 1g. Scale bar, 10 µm.

825

826   **Figure S3. Endogenous ORP5-ORP8 interaction at ER-mitochondria contact**

827   **sites.**

828   (a) Confocal images showing endogenous interaction of ORP5-8 by Duolink PLA

829   (green) in Ctrl (siCtrl), and ORP5+ORP8 (siORP5+8) knockdown HeLa cells labeled with

830   MitoTracker (red) to stain mitochondria. Images are presented as maximum projection of

831   all layers. Insets show magnifications of the boxed regions. Scale bar, 10 µm. (b) Workflow

832   for the identification of PLA signals in close proximity to the mitochondria or plasma

833   membrane. First the confocal stacks are segmented to identify the PLA foci (spots), the

834   mitochondria network (surfaces), and the plasma membrane (cells). Then 3D distance

835   maps are computed towards the outside of the surfaces (mitochondria) or inside the cells

836   allowing the measurement of the distance of each spot from the closest mitochondria or

837   membrane. Finally, PLA spots are classified into two population (red and green) based on

838   a proximity threshold of 380nm established on the precision of the detection system. (c)

839   Confocal micrographs of HeLa cell transfected with GFP-Sec22b (green) and Mito-BFP

840   (blue) and showing endogenous interaction of ORP5-8 by Duolink PLA (red). Images are

841   presented as superposition of two layers. Scale bar, 5 µm.

842

843   **Figure S4. Localization of ORP5 near SAM50-labeled mitochondria and effect**

844   **of SAM50 KD on ORP5 and Mic60 transcription.** (a) ORP5 and ORP8 full-length and

845   mutant constructs used in Fig. 4a-b and in in Fig. S4b-c. (b) EGFP-ORP5, EGFP-

846   ORP5ΔPH, EGFP-ORP5ΔORD, EGFP-ORP5ΔTM, EGFP-ORP8, EGFP-ORP8ΔPH or

847   EGFP alone were transfected in HeLa cells then immuno-precipitated from lysates and

848   analyzed by western blot using antibodies against GFP (for ORP5 or ORP8) or against

849   SAM50. (c) Confocal micrograph of a HeLa cells transfected with EGFP-ORP5 or EGFP-

850   ORP5ΔPH (green) and Mito-BFP (blue) and stained with anti-SAM50 (red) antibody.

851   Insets show magnifications of the boxed regions. Scale bar, 10 μm. (d) WB analysis

852   showing GFP (EGFP-tagged constructs), Mic60, SAM50 and Actin levels in protein

853   lysates from HeLa cells transfected with either EGFP (Control) or with EGFP-ORP5 or

854   EGFP-ORP5ΔPH constructs. (e) Quantitative RT-PCR analysis of ORP5, SAM50 and

855   Mic60 in) values of tested genes and those of reference gene (SDHA) in SAM50

856   knockdown cells versus control HeLa cells. SAM50 knockdown does not alter Mic60 RNA

857   levels and it induces an increase in ORP5 transcription. y axis: $2^{(-\Delta\Delta Ct)}$ value represents

858   differences between the mean Ct (Cycle threshold). Statistical analysis: unpaired

859   student's t-test , **$P<0.01$, ****$P<0.0001$.

860

861   **Figure S5. SAM50 regulate PLA interactions of ORP5-metaxin2 and ORP8-**

862   **Metaxin2, but not ORP5-TOM20.** (a-c) Representative confocal images showing

863   endogenous ORP5-metaxin2, ORP5-TOM20 and ORP8-metaxin2 PLA interactions

864   (green), mitochondrial network (MitoTracker, red) and nuclei (DAPI, blue) in control (siCtrl)

865   and SAM50 (siSAM50) knockdown HeLa cells. Images are displayed as single layers.

866   Scale bar, 10 μm. (d-f) Quantitative analysis of endogenous endogenous ORP5-TOM20

867   (n= 49 cells siCtrl, and n= 49 cells siSAM50) ORP5-TOM20 (n= 51ccells siCtrl, and n=52

868   cells siSAM50) and ORP5-metaxin2 (n= 35 cells siCtrl, and n= 24 cells siSAM50) PLA

869   interactions in control and SAM50 (siSAM50) knockdown HeLa cells. Data are shown as

870   mean values ±SEM. Statistical analysis were performed using unpaired student's t-test,

871   with **, $P<0.01$, n.s., not significant.

872   **Figure S6. Cristae Junctions are altered upon SAM50, Mic60 and ORP5+8**

873   **knockdowns.** (a) Representative EM micrographs showing the morphology of

874   mitochondria in HeLa cells treated with siRNA against ORP5, ORP8, Mic60 and SAM50.

875   Scale bar, 200 nm. Red arrows indicate ER elements in contact with mitochondria;

876   arrowheads indicate CJ; m, mitochondria. (b) Quantifications of the number of CJ per

877   mitochondria profile in the indicated siRNA conditions. % of CJ ±SEM, n = 170-260

878   mitochondria. Statistical analysis: unpaired student's t-test , $**P<0.01$, $***P<0.001$.

879

880   **Figure S7. ORP5 and ORP8 ORD domains mediate PS transfer between**

881   **liposomes in vitro.** (a) Coomassie stained SDS-PAGE of the recombinant ORD domain

882   of ORP5/8 proteins purified from BL21DE3 RILP cells. (b) Schematic cartoon of the in

883   vitro assay used to study ORP5/8 ORD-mediated lipid transport between liposomes. (c)

884   Donor liposomes containing fluorescent lipids (97 mol% POPC, 1 mol% TopFluor-PS, -

885   PC or -PE and 2 mol% of biotinylated-PE) and pre-bound to streptavidin beads were

886   mixed at a 1:1 molar ratio with acceptor liposomes (100 mol% POPC) in the presence or

887   absence of ORP5 or ORP8 ORD domains (250 µM of donor and acceptor liposomes and

888   0,3 µM of proteins in the reaction solution). The increase in fluorescence in the acceptor

889   liposomes, which remain unbound in the solution, was measured after 1 hour of incubation

890   at 37°C. Data are presented as % of transferred lipid ±SEM and are the mean of six

891   independent experiments. Statistical analysis: unpaired student's t-test , $***P<0.001$. (d)

892   Results of a lipid transfer experiment performed as in (c) and presented as the

893   fluorescence intensity of acceptor liposomes before (left panel) or after (right panel) their

894   floatation on a Nycodenz gradient to confirm that fluorescence comes from the liposomes

895   membrane.

896

897   **Figure S8. ORP5/8 specifically regulate levels of PS-derived mitochondrial PE**

898   **and mitochondria morphology but not PSD1 and PSS1 protein levels in situ.** (a) MS-

899   based quantification of PC and PI content (nmol/mg protein) of mitochondria isolated from

900   Ctrl, ORP5 or ORP8 knockdown cells and of Ctrl, ORP5 or ORP8 knockdown intact cells.

901   Data are shown as mean of three independent replicates ±SEM. Statistical analysis:

902   unpaired student's t-test. n.s. not significant. (b) WB analysis showing ORP5, ORP8,

903   PSD1, PSS1 and Actin levels in protein lysates from HeLa cells treated with either Ctrl

904   siRNAs or with siRNAs against ORP5 or/and ORP8. (c) Quantifications of the total

905   mitochondria surface length. Data are shown as mean of total mitochondrial surface length

906   (mm)/cell ±SD, n = 20 cell profiles and ±900 mitochondria. Statistical analysis: unpaired

907   student's t-test, n.s. not significant. (d) Quantifications of the number of mitochondria with

908   aberrant cristae morphology in the indicated siRNA conditions. Data are shown as % of

909   mitochondria ±SEM, n = 20 cell profiles and ±700 mitochondria. Statistical analysis:

910   unpaired student's t-test, **P<0.01 compared to siCtrl. (e) Schematic representation of

911   non-vesicular PS transfer mediated by ORP5/8 at ER-mitochondria contact sites. PS is

912   transported to mitochondrial membranes where it is rapidly converted into mitochondrial

913   PE.

914

915   **Figure S9. ORP5 and ORP8 knockdowns affect mitochondria respiratory**

916   **function.** (a-b) Mitochondrial oxygen consumption rate (OCR) measured in Ctrl and

917   ORP5 (a) or ORP8 (b), siRNA-treated HeLa cells. OCR trace was obtained by sequential

37

918   measurement of basal OCR (OCR$_{BAS}$), OCR after the addition of Oligomycin, OCR after

919   the addition of FCCP (OCR$_{FCCP}$) and OCR after the addition of Rotenone/Antimycin A.

920   Note the reduced OCR in siORP5 and siORP8 cells compared to Ctrl siRNA cells. Error

921   bars denote ±SEM. Data shown in the bar charts are the mean of 4 independent repeats

922   (n=4). Statistical analysis: unpaired student's t-test, *$P<0.05$, **$P<0.01$ compared to Ctrl.

923

924   **STAR METHODS**

925   **RESOURCES AVAILABILITY**

926   **Lead contact**

927   Further information and requests for resources and reagents should be directed to

928   and will be fulfilled by the lead contact, Francesca Giordano

929   (francesca.giordano@i2bc.paris-saclay.fr). All unique reagents generated in this

930   study are available from the Lead Contact.

931   **Materials availability**

932   All unique reagents generated in this study are available from the Lead Contact.
933

934   **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

935   **Cell line**

936   HeLa cells were used for KD, transfection, biochemistry, light and electron

937   microscopy, and lipidomic studies. HeLa cells were cultured in Dulbecco's Modified

938   Eagle Medium (DMEM, Life Technologies) supplemented with 10% FBS (Life

939   Technologies) and 1% penicillin/streptomycin (P/S, 100 units/ml penicillin and

940   10 μg/mL streptomycin, Life Technologies) and maintained at 37°C with 5% CO_2.

941
942   **METHOD DETAILS**

943   **cDNA plasmids and molecular cloning**

944   EGFP-ORP5, EGFP-ORP8, EGFP-ORP5ΔPH, EGFP-ORP8ΔPH, EGFP-

945   ORP5ΔORD and EGFP-ORP5ΔTM were described in (Galmes et al., 2016). RFP-

946   Sec22b (Gallo, Danglot et al., 2020). The following reagents were kind gifts: GFP-

947   Sec61β from T. Rapoport (Harward University)(Shibata, Voss et al., 2008), PHPLCδ-

948   RFP (Chung et al., 2015); Mito-BFP (Addgene: # 49151); ssHRP-KDEL from T.

949   Schikorski (Schikorski, Young et al., 2007); GST-ORD8 (ORD ORP8, corresponding

950   to aa 328-767) from P. De Camilli (Chung et al., 2015).

951   Cloning of HA-ORP5, Mic60-GFP and GST- ORD5 (ORD ORP5) : cDNAs of

952   ORP5 (full-length), Mitoflin (full-length from FLAG- Mic60 (Ott et al., 2015) and GST-

953   ORD5 (corresponding to aa 265-703), were amplified by PCR. In all PCR reactions,

954   Herculase II fusion DNA polymerase (Agilent) was used.

955   Primers used were (coding sequence shown in lowercase):

956   5' AgeI-HA-ORP5_Fw GGCGGC *ACCGGT* cgccacc ATGTACCCATACGATGTTCCA

957   GATTACGCT atgaaggaggaggccttcctc

958   3' XhoI-STOP-ORP5_Rv GGC *CTCGAG* ctatttgaggatgtggttaatg

959   5' KpnI- Mic60_Fw AGACCCAAGCTT *GGTACC* atg

960   3' BamHI-GC- Mic60_Rv GTAATC *GGATTC* GC ctctggct

961   5' SalI-TC-ORD5_Fw GCACAG GTCGAC TC gagacccctggggccccggt

962   3' NotI-STOP-ORD5_Rv GCACA *GCGGCCGC* ctactgtggccggagggctggtcg

963   For the HA-ORP5 cloning the PCR product (carrying the HA tag at the N-

964   terminus of ORP5) was ligated between AgeI and XhoI in the pEGFP-C1 vector

965   (Clontech) and replacing the GFP- with the HA-tag. For the other clonings the PCR

966   products were ligated between KpnI and BamHI for Mic60, and between SalI and

39

967   NotI for ORD5, in the pEGFP-N1 vector (Clontech) to generate Mic60-EGFP or in the

968   pGEX-6P-1 to generate GST-ORD5.

969   **Cell transfection**

970   HeLa cells were seeded in 13mm glass coverslips (Agar Scientific) or in 10 cm

971   dishes and were cells were transfected with 1 μg or 24 ug DNA, respectively, using

972   Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. Cells

973   were cultured for 24 hours prior to analysis.

974   **RNA interference**

975   ORP5, ORP8, SAM50 and Mic60 KD in Hela cells was achieved by siRNA

976   transfection. HeLa cells were seeded in 13 mm coverslips (Agar Scientific) or in 10

977   cm dishes transfected with 200 nM siRNA using Oligofectamine (Invitrogen) following

978   the manufacturer's instruction. Cells were cultured for 48 hours prior to analysis.

979

980   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)
SAMM50	Dharmacon, J-017871-18
SAMM50	Dharmacon, J-017871-19
Non-targeting	Dharmacon, D-001810-10
Mic60	5'AAUUGCUGGAGCUGGCCUUTT-3' (John, Shang et al., 2005)

981

982

983

984   **mRNA analyses by quantitative reverse transcriptase PCR (qPCR)**

985   Total RNA was isolated from HeLa cells transfected with siRNAs for 48 hours as

986   described above, by using a Purelink™ kit (Ambion/Thermo Scientific, Foster City,

987   CA). The RNA (0.5 µg per specimen) was reverse transcribed with a SuperScript

988   VILO™ cDNA synthesis kit (Invitrogen/Thermo Scientific, Carlsbad, CA) according to

989   the manufacturer's protocol. Quantification of the mRNAs of interest was carried out

990   on a Roche Lightcycler™ 480 II instrument by using SYBR-Green I Master mix (Roche,

991   Basel, Switzerland) and primers specified in the Table bellow. Succinate

992   dehydrogenase complex. subunit A, the mRNA of which remained markedly stable

993   under the present conditions, was employed as a reference housekeeping mRNA.

994   Relative mRNA levels were calculated by using the $-\Delta\Delta$Ct method.

995   Sequences of the primers used for qPCR:

mRNA	Sequence 5'-3'
SDHA* (housekeeping)	Fw: CATGCTGCCGTGTTCCGTGTGGG
	Rv:GGACAGGGTGTGCTTCCTCCAGTGCTCC
ORP5	Fw: GTGCCGCTGGAGGAGCAGAC
	Rv: AGGGGCTGTGGTCCTCGTATC
SAMM50	Fw: CAAGTGGACCTGATTTTGGAGG
	Rv: AGACGGAGCAATTTTTCACGG
Mic60	Fw: GTTGTATCTCAGTATCATGAGCTGG
	Rv: GTTCAGCTGATCAATACGACGA

996   *succinate dehydrogenase complex, subunit A

997

998   **Immunoprecipitation of ORPs**

999   HeLa cells transfected with EGFP-tagged ORPs were washed in cold PBS and

1000   lysed on ice in lysis buffer [50 mM Tris, 120 mM NaCl, 40 mM Hepes, 0.5% digitonin,

41

1001 0.5% CHAPS, pH 7.36, and protease inhibitor cocktail (Roche)]. Cell lysates were then

1002 centrifuged at 21 000 g for 20 min at 4°C. Supernatants were then incubated with

1003 Chromotek GFP-trap agarose beads (Allele Biotech) for 1 hour at 4°C under rotation.

1004 Subsequently beads were washed in 0.1 M phosphate buffer. After extensive washes

1005 in cold lysis buffer, immunoprecipitated proteins bound to the beads were processed

1006 for Mass Spectrometry analysis or incubated in sample buffer (containing 2% SDS)

1007 and then boiled for 1 min at 97 °C. In the latter case immunoprecipitates were loaded

1008 and separated in 10% SDS–PAGE gel and immunoblotting was carried out.

1009

1010 **Western blotting**

1011 For immunoblotting, cells were resuspended in lysis buffer [50 mM Tris, 150 mM NaCl,

1012 1% Triton X-100, 10 mM EDTA, pH 7.2, and protease inhibitor cocktail (Roche)]. Cell

1013 lysates were then centrifuged at 21 000 g for 20 min at 4°C. The supernatants were

1014 boiled in reducing SDS sample buffer. Proteins isolated from HeLa cells or obtained

1015 by immunoprecipitation were were subjected to SDS-PAGE gels for electrophoresis

1016 separation. The separated proteins were transferred to 0.45 µm Nitrocellulose

1017 membrane (GE Healthcare). The membrane was blocked by 5% non-fat milk in TBST

1018 buffer (TBS buffer with 0.1% Tween 20) for 1 h at room temperature and washed 3

1019 times, for 5 min each, with TBST. Then the membrane was incubated with the primary

1020 antibodies (antibodies and dilutions listed below) at 4°C overnight. The membrane

1021 was washed 3 times wash with TBST incubated with the peroxidase-conjugated anti-

1022 rabbit IgG secondary antibody (1:5000, in 5%milk in TBST) or anti-mouse IgG

1023 secondary (1:5000, in 5%milk in TBST) at room temperature for 1h, followed with

1024 washing and detection using the enhanced chemiluminescence (ECL) detection kit

1025 (Cytiva). For Western blot quantification, bands of protein of interest were detected

1026   using ChemiDoc™ Imaging Systems (Life Science Research, Bio-Rad) and analyzed

1027   using Image Lab™ Software. All data are presented as mean ± standard error of the

1028   mean (SEM) of three experimental replicates.

Primary Antibodies (Company & Reference)	Dilution
IP3R-3 (BD Transduction Laboratories, 610312)	1:2000
ORP5 (SIGMA, HPA038712)	1:1000
ORP5 (SIGMA, HPA058727)	1:1000
β-Actin (Abcam, ab8226)	1:1000
ORP8 (GeneTex, GTX121273)	1:1000
SAM50 (SIGMA, HPA034537)	1:1000
Mic60 (Proteintech, 10179-1-AP)	1:1000
metaxin2 (Santa Cruz, sc-514231)	1:1000
TOM20 (BD Transduction Laboratories, 612278)	1:10000
PDI (GeneTex, GTX30716)	1:500
VAPB (SIGMA, HPA013144)	1:500
PSS1 (PTDSS1, SIGMA, HPA016852)	1:300
PISD (SIGMA, HPA031090)	1:300
GFP (Roche, 11814460001)	1:1000
Secondary Antibodies (Company & Reference)	Dilution
anti-mouse-HRP (GE Healthcare, NA931V)	1:10000
anti-rabbit-HRP (GE Healthcare, NA934V)	1:10000

1029

1030   **Mass spectrometry-proteomic analysis**

1031   Mass Spectrometry (MS) analysis was carried out by the proteomics/mass

1032   spectrometry platform in IJM (http://www.ijm.fr/plateformes/spectrometrie-de-masse).

1033   Briefly, after washes with binding buffer, immunoprecipitations beads were rinsed with

43

1034   100 µl of NH4HCO3 25 mmol/l. Proteins on beads were digested overnight at 37°C by

1035   sequencing grade trypsin (12.5 µg/ml; Promega Madison, Wi, USA) in 20 µl of

1036   NH4HCO3 25 mmol/l. Digests were analysed by an Orbitrap Fusion (Thermo Fisher

1037   Scientific, San Jose, CA) equipped with a Thermo Scientific EASY-Spray

1038   nanoelectrospray ion source and coupled to an Easy nano-LC Proxeon 1000 system

1039   (Thermo Fisher Scientific, San Jose, CA). MS/MS data were processed with Proteome

1040   Discoverer 1.4 software (Thermo Fisher scientific, San Jose, CA) coupled to an in

1041   house Mascot search server (Matrix Science, Boston, MA; version 2.4.2). MS/MS

1042   datas were searched against SwissProt databases with Homo sapiens taxonomy.

1043   The Mascot score for a protein is the summed score for the individual peptides, e.g.

1044   peptide masses and peptide fragment ion masses, for all peptides matching a given

1045   protein. For a positive protein identification, the mascot score has to be above the 95%

1046   confidence level. In Mascot, the ions score for an MS/MS match is based on the

1047   calculated probability, P, that the observed match between the experimental data and

1048   the database sequence is a random event. The reported score is $-10\text{Log}_{10}(P)$. A score

1049   of 200 indicates a probability of 10^{-20}. Scores greater than 70 are significant, while

1050   scores lower than 40 should not be considered or carefully validated at MS/MS level

1051   (source: http://www.matrixscience.com/help/interpretation_help.html). We thus set up

1052   a Mascot score threshold of 50.

1053

1054   **Cell fractionation**

1055   HeLa cells ($100\text{x}10^6$ cells) were harvested 48 hours after transfection with siRNA

1056   oligos and washed with PBS by centrifugation at 600 g for 5 min. The cell pellet was

1057   resuspended in starting buffer (225 mM mannitol, 75 mM sucrose and 30 mM Tris-HCl

1058   pH 7.4) and homogenized using a Tissue Grinder Dura-Grind®, Stainless Steel,

44

1059 Dounce (Wheaton). The homogenate was centrifuged three times at 600 g for 5 min

1060 to remove nuclei and unbroken cells. The crude mitochondria was pelleted by

1061 centrifugation at 10 000 g for 10 min. To separate MAM and pure mitochondria

1062 fractions, the pellet was resuspended in MRB buffer (250 mM mannitol, 5 mM HEPES

1063 and 0.5 mM EGTA, pH 7.4) and layered on top of different concentrations of Percoll

1064 gradient (225 mM mannitol, 25 mM HEPES, 1 mM EGTA pH 7.4 and 30% or 15%

1065 Percoll). After centrifugation at 95 000 g for 30 min, two dense bands containing either

1066 the pure mitochondria or MAM fraction were recovered and washed twice with MRB

1067 buffer by centrifugation at 6300 g for 10 min to remove residual Percoll and residual

1068 contamination. MAM was pelleted by centrifugation at 100 000 g for 1 hour. MAMs and

1069 pure mitochondria pellets were resuspended in Lysis Buffer (50 mM Tris, 150 mM

1070 NaCl, 1% Triton X-100, 10 mM EDTA, pH 7.2, and protease inhibitor cocktail) and

1071 protein concentrations were determined by Bradford assay. Equal amount of proteins

1072 were loaded on 4-20% gradient SDS-PAGE gels (Biorad) and immunoblotting was

1073 carried out. Pure mitochondria were processed for MS-lipidomic analysis.

1074

1075 **Mass Spectrometry-lipidomic analysis**

1076 700 µl of homogenized cells were mixed with 800 µl 1 N HCl:CH$_3$OH 1:8 (v/v), 900

1077 µl CHCl$_3$ and 200 µg/ml of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT;

1078 Sigma Aldrich). The organic fraction was evaporated using a Savant Speedvac

1079 spd111v (Thermo Fisher Scientific). Lipid pellets were reconstituted in running solution

1080 (CH$_3$OH:CHCl$_3$:NH$_4$OH; 90:10:1.25; v/v/v). Phospholipid species were analyzed by

1081 electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid triple

1082 quadrupole/linear ion trap mass spectrometer (4000 QTRAP system; Applied

1083 Biosystems SCIEX) equipped with a TriVersa NanoMate (Advion Biosciences) robotic

1084 nanosource. Phospholipid profiling was executed by (positive or negative) precursor

1085 ion or neutral loss scanning at a collision energy of 35 eV for neutral loss 141

1086 (phosphatidylethanolamine (PE)). Phospholipid quantification was performed by

1087 multiple reaction monitoring (MRM), the transitions being based on the neutral losses

1088 or the typical product ions as described above. The MRM dwell time was set to 100

1089 ms and typically the signal was averaged over 20 cycles. Lipid standards used were

1090 PE25:0 and PE43:6 (Avanti Polar Lipids). The data were corrected for isotope effects

1091 as described by (Liebisch, Lieser et al., 2004).

1092

1093 **Immunofluorescence**

1094   HeLa cells were seeded on 13 mm glass bottom coverslips (Agar Scientific). KD

1095 or/and transfected cells were incubated with 1 μM MitoTracker (mitochondrial marker,

1096 Invitrogen) for 30 min at 37°C, 5% CO_2 and then fixed with 4% PFA/PBS for 15 min at

1097 room temperature. Fixed cells were then washed in PBS and incubated with 50 mM

1098 NH4Cl/PBS for 15 min at room temperature. After washing with PBS and blocking

1099 buffer (1% BSA/ 0.1% Saponin in PBS), cells were incubated with primary antibodies

1100 diluted in blocking buffer for 1 hour at room temperature and then with fluorescently-

1101 labeled secondary antibodies. After washing with blocking buffer and then PBS,

1102 coverslips were mounted on microscopy slides and images were acquired on Confocal

1103 inverted microscope SP8-X (DMI 6000 Leica). Optical sections were acquired with a

1104 Plan Apo 63x oil immersion objective (N.A. 1.4, Leica) using the LAS-X software.

1105 Fluorescence was excited using either a 405nm laser diode or a white light laser, and

1106 later collected after adjusting the spectral windows with GaAsP PMTs or Hybrid

1107 detectors. Images from a mid-focal plane are shown. Images were processed and

1108 fluorescence was analysed off line using Image J. For co-localization analysis of

46

1109 fluorescent signals, the acquired images were processed using the JACoP plugin in

1110 ImageJ to assess the Pearson's correlation coefficient. The obtained values, ranging

1111 from 0 to 1 (1=max correlation), indicated the association between the signals

1112 analysed. To assess the distance between ORP5 or ORP8 and mitochondria confocal

1113 images were processed by Imaris, using a similar approach described in the PLA

1114 section.

1115

Primary Antibody (Company, Reference)	Dilution
ORP5 (SIGMA, HPA038712)	1:150
ORP5 (SIGMA, HPA058727)	1:150
ORP8 (Santa Cruz, sc-134408)	1:200
ORP8 (GeneTex, GTX121273)	1:200
SAM50 (SIGMA, HPA034537)	1:100
HA (SIGMA, H3663)	1:800
Secondary Antibody (Company, Reference)	**Dilution**
Alexa Fluor 488 anti-rabbit (Invitrogen, A-11008)	1:500
Alexa Fluor 488 anti-mouse (Invitrogen, A-21121)	1:500
Alexa Fluor 555 anti-mouse (Invitrogen, A-21422)	1:500

1116

1117 ***In situ* proximity ligation assay (PLA)**

1118 The protein-protein interactions in fixed HeLa cells were assessed using *in situ*

1119 PLA (Duolink®SIGMA) according with the manufacturer's instructions. Briefly, HeLa

1120 cells seeded on 13 mm glass coverslips (Agar Scientific) were incubated with

1121 MitoTracker Red (mitochondrial marker, Invitrogen, 1 μM in DMEM) for 30 min at 37°C

1122 or co-transfected with PHPLCd-RFP (plasma membrane marker) plus Mito-BFP

1123 (mitochondrial marker). Cells were thereafter fixed with 4% PFA for 30 minutes at room

47

1124 temperature and incubated with primary antibodies (dilutions in the table below) in

1125 blocking solution (1% BSA, w/v 0.01% saponin, w/v, in PBS) for 1h at room

1126 temperature. PLUS and MINUS PLA probes (anti-murine and anti-rabbit IgG

1127 antibodies conjugated with oligonucleotides, 1:5 in blocking solution) were then

1128 incubated with the samples for 1h at 37°C. Coverslips were thereafter washed in 1x

1129 wash buffer A and incubated with ligation solution (5x Duolink$^®$ Ligation buffer 1:5,

1130 ligase 1:40 in high purity water) for 30 min at 37°C. After the ligation step, cell samples

1131 were washed in 1x wash buffer A and incubated with the polymerase solution (5x

1132 Amplification buffer 1:5, polymerase 1:80 in high purity water) for 1h40min at 37°C.

1133 Polymerase solution was washed out from the coverslips with 1x wash buffer B and

1134 0.01x wash buffer B. Vectashield Mounting Medium with or without DAPI (Vector

1135 Laboratories) was used for mounting. Images were acquired on Confocal inverted

1136 microscope SP8-X (DMI 6000 Leica). Optical sections were acquired with a Plan Apo

1137 63x oil immersion objective (N.A. 1.4, Leica) using the LAS-X software. Fluorescence

1138 was excited using either a 405nm laser diode or a white light laser, and later collected

1139 after adjusting the spectral windows with GaAsP PMTs or Hybrid detectors. Images

1140 from a mid-focal plane or maximal projection of all layers are shown. Images were

1141 processed and the number and the distance of PLA dots to mitochondria and to the

1142 plasma membrane were assessed using the Imaris software (v 9.3, Bitplane). Briefly,

1143 segmented 3D images (PLA foci identified as "spots", mitochondria identified as

1144 "surfaces", and plasma membrane represented as "cell" were generated from confocal

1145 Z-stack images and the shortest distance between each spot center and the nearest

1146 point of the surface or cell object was calculated based on a 3D distance map. Spots

1147 objects (PLA dots) with a distance smaller than 380nm from surfaces (mitochondria)

1148 and cell (plasma membrane) objects were considered at a close proximity of these

1149 objects. The threshold of 380 nm was used as an estimation of the PLA reaction

1150 precision including both primary and secondary antibodies (30nm) plus half the FWHM

1151 of the PLA amplification signals (350nm).

1152

Pair of Antibodies (Company, Reference)	Dilution
ORP5 (SIGMA, HPA038712) ORP8 (Santa Cruz,sc-134408)	1: 150 1: 150
ORP8 (Santa Cruz,sc-134408) SAM50 (SIGMA, HPA034537)	1:150 1:200
ORP5 (SIGMA, HPA038712) metaxin2 (Santa Cruz, sc-514231)	1:150 1:150
ORP8 (GeneTex, GTX121273) metaxin2 (Santa Cruz, sc-514231)	1:150 1:150
ORP5 (SIGMA, HPA038712) TOM20 (BD Transduction Laboratories, 612278)	1:150 1:150
ORP8 (GeneTex, GTX121273) TOM20 (BD Transduction Laboratories, 612278)	1:150 1:150

1153

1154 **Electron Microscopy Analysis**

1155 *Conventional EM:* For conventional EM, cells grown on 13 mm glass bottom

1156 coverslips (Agar Scientific) were fixed with 2.5% glutaraldehyde and 2% PFA in 0.1 M

1157 cacodylate, 0.05% $CaCl_2$ buffer for 24 hours. After several washes with 0.1 M

1158 cacodylate buffer, the cells were postfixed with 1% OsO_4, 1.5% potassium ferricyanide

1159 in 0.1M Cacodylate for 1 hour. After several washes with 0.1 M cacodylate buffer and

1160 H_2O, the cells were stained with 0.5% uranyl acetate for 24 hours. After several washes

1161 with H_2O, the cells were dehydrated in ethanol and embedded in Epon while on the

1162 coverslips. Ultrathin sections were prepared, counterstained with uranyl acetate and

1163 observed under a MET JEOL 1400 equipped with a Orius High speed (Gatan) camera.

1164   ***HRP Detection:*** HeLa cells expressing HRP-KDEL were fixed on coverslips with

1165   1.3% glutaraldehyde in 0.1 M cacodylate buffer, washed in 0.1 M ammonium

1166   phosphate [pH 7.4] buffer for 1 hour and HRP was visualized with 0.5 mg/ml DAB and

1167   0.005% H_2O_2 in 0.1 M Ammonium Phosphate [pH 7.4] buffer. Development of HRP

1168   (DAB dark reaction product) took between 5 min to 20 min and was stopped by

1169   extensive washes with cold water. Cells were postfixed in 2% OsO_4+1% $K_3Fe(CN)_6$ in

1170   0.1 M cacodylate buffer at 4°C for 1 hour, washed in cold water and then contrasted in

1171   0.5% uranyl acetate for 2 hours at 4°C, dehydrated in an ethanol series and embedded

1172   in epon as for conventional EM. Ultrathin sections were counterstained with 2% uranyl

1173   acetate and observed under a FEI Tecnai 12 microscope equipped with a CCD (SiS

1174   1kx1k keenView) camera.

1175   ***Immunogold labelling:*** HeLa cells were fixed with a mixture of 2%PFA and

1176   0.125% glutaraldehyde in 0.1 M phosphate buffer [pH 7.4] for 2 hours, and processed

1177   for ultracryomicrotomy as described previously (Slot & Geuze, 2007). Ultrathin

1178   cryosections were single- or double-immunogold-labeled with antibodies and protein

1179   A coupled to 10 or 15 nm gold (CMC, UMC Utrecht, The Netherlands), as indicated in

1180   the legends to the figures. Immunogold-labeled cryosections were observed under a

1181   FEI Tecnai 12 microscope equipped with a CCD (SiS 1kx1k keenView) camera.

1182

Antibodies (Company, Reference)	Dilution
GFP (Life technologies, A11122)	1:100
HA (SIGMA, H3663)	1:500
PDI (Genetex, GTX30716)	1:500

1183

1184   For the quantification of the number of cristae junction in Epon sections, about 200

1185   mitochondria were analyzed in randomly selected cell profiles and cristae junctions

1186 were counted in each of the mitochondria profile and reported as number of

1187 cristae/mitochondria profile. All data are presented as mean ±SEM of three

1188 experimental replicates.

1189 For the quantification of ER-mitochondria contact sites in HRP-stained Epon

1190 sections, the total circumference of each mitochondria and the length of the multiple

1191 HRP-positive ER segments closely associated (<30 nm) with them were measured by

1192 manual drawing using the iTEM software (Olympus), as in (Galmes et al., 2016,

1193 Giordano et al., 2013), on acquired micrographs of HeLa cells for each cell profile, as

1194 indicated in the figure legends. Cells were randomly selected for analysis without prior

1195 knowledge of transfected plasmid or siRNA. All data are presented as mean (%) ±SEM

1196 of three experimental replicates.

1197 For the quantifications of the total mitochondria surface the total circumference of

1198 each mitochondria was measured by manual drawing using the iTEM software

1199 (Olympus), and data are shown as average of total mitochondrial surface length

1200 (μm)/cell $\pm$SD of three experimental replicates.

1201 For the quantification of ORP5 immunogold labeling on ultrathin cryosections, 150

1202 gold particles localized at ER-mitochondria contact sites were counted on acquired

1203 micrographs of randomly selected cell profiles at specific ranges of distance from CJ

1204 (0-50, 50-100, 100-150, 150-200 nm) in each of three experiments. All data are

1205 presented as mean (%) ±SEM of three technical replicates.

1206

1207 **ORP5 and ORP8 ORD domain purification**

1208 Escherichia coli BL21DE3 RILP (Invitrogen) cells were transformed with plasmids

1209 encoding for GST tagged ORP5 (aa 265-703) or ORP8 (aa 328-767) ORD domains

1210 following the manufacturer's instruction. Bacteria were then grown overnight at 37°C

51

1211 and used to inoculate a large-scale volume (1L). When the OD_{600} reached 0.4, cultures

1212 were cooled down and incubated at 18°C until they reached O_{D600} = 0.65. Cells were

1213 induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration

1214 of 0.1 mM and incubated overnight at 18°C before harvesting. Cells were resuspended

1215 in 35 mL binding buffer (1X PBS, 1 mM EDTA, 1 mM DTT, Protease inhibitor) then 250

1216 units of benzonase nuclease (Sigma) were added to the resuspension. Cells were

1217 lysed by sonication and the supernatant was recover after 20 min centrifugation at 184

1218 000g and 4°C. Supernatant containing GST tagged proteins was incubated with 2 mL

1219 of Glutathione Sepharose 4 fast flow for 1 hour at 4°C under nutation. Beads were

1220 washed using a series of wash buffers: 1st (1X PBS, 1 mM EDTA, 1 mM DTT), 2nd

1221 HSP-removal buffer (50 mM Tris pH 7.5, 50 mM KCl, 20 mM $MgCl_2$, 5 mM ATP) then

1222 cleavage buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). Cleavage

1223 of the GST tag was realized overnight at 4°C using Prescission protease. Untagged

1224 proteins were eluted with cleavage buffer, flash frozen and stored at -80°C until lipid

1225 transfer assay was performed.

1226

1227 **Liposome preparation**

1228 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-

1229 glycero-3-phosphoethanolamine-N-(cap biotinyl) (Biotinyl Cap PE), 1-palmitoyl-2-

1230 (dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphoethanolamine

1231 (TopFluor-PE), 1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-

1232 3-phospho-L-serine (TopFluor-PS), 1-palmitoyl-2-(dipyrrometheneboron

1233 difluoride)undecanoyl-sn-glycero-3-phosphocholine (TopFluor-PC) were purchased

1234 from Avanti Polar Lipids as chloroform solutions.

52

1235   1 μmol of the appropriate lipid mixtures in chloroform solution was dried in a glass

1236   tube for 10 min under a gentle stream of argon, and then for 1 hour under vacuum.

1237   The dried lipid films were resuspended in 1 mL of buffer H (25 mM HEPES/KOH, pH

1238   7.7; 150 mM KCl; 10%(v/v) Glycerol) by vigorously vortexing for 30 min at room

1239   temperature. Unilamellar liposomes were produced by seven freeze-thaw cycles (30

1240   sec in liquid nitrogen followed by 5 min in a 37°C water bath) and extrusion (at least

1241   21 times) through a polycarbonate filter with 100 nm pore size (polycarbonate

1242   membranes from Avanti Polar Lipids). The liposomes were then stored on ice.

1243

1244   **Lipid Transfer assay *in vitro***

1245   The lipid transfer assays were realized with liposomes prepared as described

1246   above. The donor liposomes contained 1% mol TopFluor lipids (-PS, -PC or -PE) and

1247   2% mol Biotinyl Cap PE and 97 mol% POPC. The acceptor liposomes contained only

1248   POPC. For each reaction, 25 μL of streptavidin-coated magnetic beads

1249   (DynabeadsMyOne Streptavidin T1, Invitrogen) were washed in buffer H and mixed

1250   with 25 μL of 1 mM donor liposomes. The mixture was incubated for 1 hour at 25°C

1251   with intermittent gentle mixing. Bead-bound donor liposomes were then washed,

1252   resuspended in 25 μL and mixed with 25 μL of 1 mM acceptor liposomes and 50 μL of

1253   buffer H or protein (0.3 μM protein and 2.5 μM TopFluor lipids in the reaction solution).

1254   The mixture was incubated at 37°C for 1 hour with intermittent gentle mixing.

1255   Supernatant containing acceptor liposomes was recovered after binding of bead-

1256   bound donor liposomes to a magnetic rack. TopFluor fluorescence of acceptor and

1257   donor liposomes was measured (after solubilization with 0.4% (w/v) n-dodecyl-β-D-

1258   maltoside, DDM) in a SpectraMax M5 plate reader (Molecular Device) equilibrated to

1259   30°C (excitation: 450 nm; emission: 510 nm; cutoff: 475 nm; low gain). To confirm that

1260　fluorescence was transferred to acceptor liposomes, a fraction of the reaction

1261　supernatant – which has not been solubilized with DDM – was floated on a Nycodenz

1262　density gradient. 50 µL of supernatant was mixed with 100 µL of buffer H and 150 µL

1263　of Nycodenz 80% in buffer H. The solution was transferred to a 0.8 mL Ultra-Clear

1264　centrifuge tube (Beckman Coulter) and overlaid with 250 µL of Nycodenz 30% in buffer

1265　H and 75 µL of buffer H. The tubes were centrifuged in a SW 55 Ti rotor (Beckman

1266　Coulter) at 246,000 g for 4 hours at 4 °C. 50 µL were collected from the top of the

1267　gradient and the fluorescence was measured. The percentage of lipids transferred

1268　from donor to acceptor liposomes in the presence of ORD5 and ORD8 was determined

1269　using the following formula: $100*F_{acceptor}/(F_{acceptor}+F_{donor})$.

1270

1271　**Radiometric assay for the conversion of PS to PE *in situ***

1272　*Radiometric assays without drugs:*

1273　　　　Hela cells were seeded on 6-well plates and transfected for 48 hours with the non-

1274　targeting, ORP5 or ORP8-specific siRNAs specified above by using Oligofectamine

1275　(Thermo Fisher Scientific). The cells were then washed and shifted into Hanks

1276　balanced salt solution (Gibco) supplemented with a serine-free MEM amino acid

1277　mixture and MEM vitamins (Gibco), followed by 18 hours labeling with 2 µl/well L-

1278　[^{3}H(G)]serine (30.9 Ci/mmol, NET24800, Perkin-Elmer)(Fig. 6c). After the labeling (Fig.

1279　6c) or the chase (Fig. 6d), the cells were scraped into 0.9 ml 2% NaCl per well, a 0.1

1280　ml aliquot was withdrawn for protein analysis with the BCA assay (Thermo Fisher

1281　Scientific), and, after adding 50 nmol of unlabeled PS as carrier, the remaining 0.8 ml

1282　was subjected to lipid extraction by an acid modification of the Folch method (Kim,

1283　Song et al., 2017). After drying, the lipids were resolved in 50 µl CHCl$_3$ and applied on

1284　Merck TLC Silica gel 60^{TM} plates, followed by separation by using CHCl$_3$-methanol-

54

1285 acetic acid-H2O (50:30:8:3.5) as solvent. The PS and PE spots identified from the

1286 mobility of standards run on the same plates were scraped into scintillation vials for

1287 analysis of [^{3}H] radioactivity. The DPM values were corrected for total cell protein, and

1288 the ratio of [^{3}H] in PE vs. PS calculated.

1289 *Radiometric assays with drugs:*

1290 Post 48hr incubation of the cells with transfection complex, media was removed and

1291 cell monolayer was washed once with PBS and once with serum-free DMEM. Cells

1292 were incubated in a serum-free medium for 12 h. In some experiments, either β-chloro-

1293 L-alanine (1 mM) or Hydroxylamine (5 mM) was added to serum-free DMEM, 30 mins

1294 prior to pulse, i.e after 11 hr 30 mins serum starvation, cells were starved in serum-

1295 free DMEM containing the inhibitor for another 30 mins. Cells were washed twice with

1296 PBS, and then incubated for 1 hr at 37 °C, CO_2 incubator in HBSS media containing

1297 1X MEM amino acids and 1X MEM vitamins containing 7 μCi/ml of [^{3}H]serine. After the

1298 pulse, the cells were washed twice with PBS and chased for 4 hr in serum-free DMEM

1299 at 37 °C. The chase media also contained β-chloro-L-alanine (1 mM)

1300 or Hydroxylamine (5 mM). Samples were then washed twice with ice-cold PBS,

1301 scraped into 2% NaCl, and the lipids were extracted according to Bligh and Dyer. A

1302 fraction of each sample was lysed for protein estimation and blot as described above.

1303

1304 **Mitochondrial respiration assay**

1305 Oxygen Consumption rate (OCR) was measured using the XF$_p$ Extracellular Flux

1306 Analyzer (Seahorse Bioscience Inc.). HeLa cells were seeded on a 6-well plate 3 days

1307 before the Seahorse experiment and KD of the proteins of interest was realized 2 days

1308 before. The day after KD, HeLa cells transfected with Ctrl, ORP5, or ORP8 siRNAs

1309 were plated in a Seahorse XFp 8-mini wells microplate. 20,000 HeLa cells were

55

1310   seeded in each well (except in the blank wells used for the background correction) in

1311   180 µl of culture medium, and incubated overnight at 37℃ in 5% CO2. One day after,

1312   the culture medium was replaced with 180 µl of XF DMEM Medium Solution pH 7.4

1313   and then the 8-mini wells microplate was moved in a 37°C non-CO2 incubator before

1314   measurement. OCR was determined before drug additions and after addition of

1315   Oligomycin (1.5 µM), Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP,

1316   0.5 µM), and Rotenone/Antimycin A (0.5 µM) (purchased from Agilent). After each

1317   assay, all the raw OCR data were analyzed using WAVE software.

1318

1319   **Quantifications and Statistical Analysis**

1320   Statistical analysis was performed with Microsoft Excel or GraphPad Prism 9.0.

1321 The data were presented as mean $\pm$ SEM. The n, indicated in the figures and figure

1322 legends, represent the total number of cells analyzed in three or more biological

1323 replicates, as stated in the figures legend. Statistical significance of two data sets

1324 were determined by unpaired student's *t*-test, with * $p<0.05$, ** $p<0.01$, *** $p<0.001$

1325 and **** $p<0.0001$.

1326

1347

1348   **AUTHOR CONTRIBUTIONS**

1349   FG conceived and supervised the work. VO designed and supervised the

1350   radiometric assays for PS-to-PE conversion and the expression analysis by RT-PCR.

1351   DT designed and supervised the *in vitro* lipid transfer assays. LR performed and

1352   analyzed the cell experiments including cell imaging and Seahorse analysis. VC

1353   performed and analyzed most of the cell experiments including immunofluorescence

1354   for endogenous proteins and Duolink. LR, AH, and FG performed and analyzed the

1355   EM experiments. CS participated to the setting up of the Seahorse experiments. CS,

1356   AH and DT performed and analyzed the *in vitro* lipid transfer assays. AA, EJ and AK

1357   performed and analyzed the radiometric assays for PS-to-PE conversion. EM, JD and

1358   JS performed MS-lipidomic analysis. RLB provided tools and techniques for Duolink

1359   imaging analysis. JN and NEK provided technical help in western blot analysis and

57

1360   generated some of the constructs for mammalian cell expression. VC, LR and FG

1361   wrote the manuscript and all authors commented on the manuscript.

1362

1363   **CONFLICT OF INTERESTS**

1364   The authors declare that they have no competing interests.

1365

1366   **DATA AVAILABILITY SECTION**

1367   This study includes no data deposited in external repositories.

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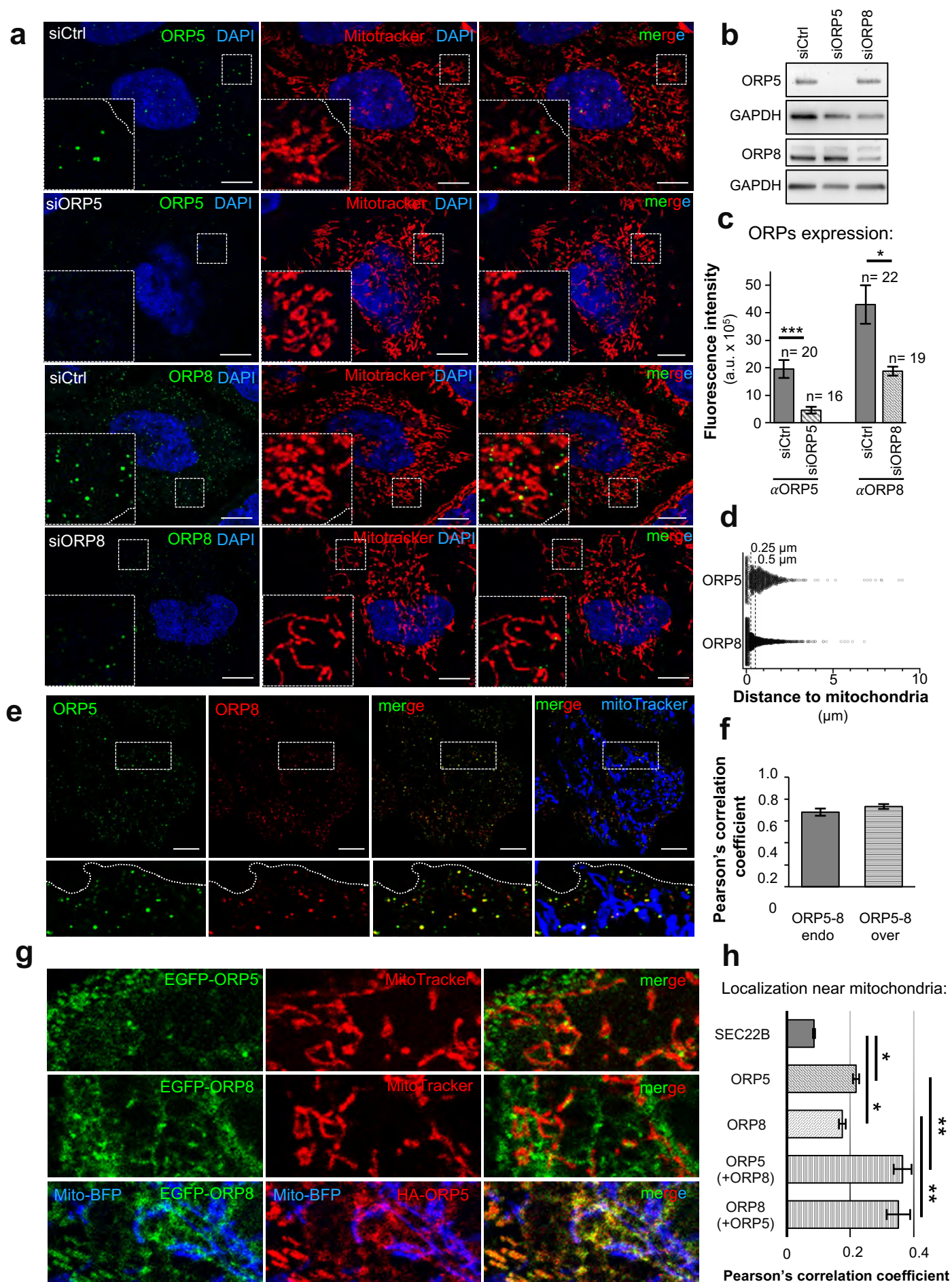1516   associated ER membranes. EMBO reports 22: e51323
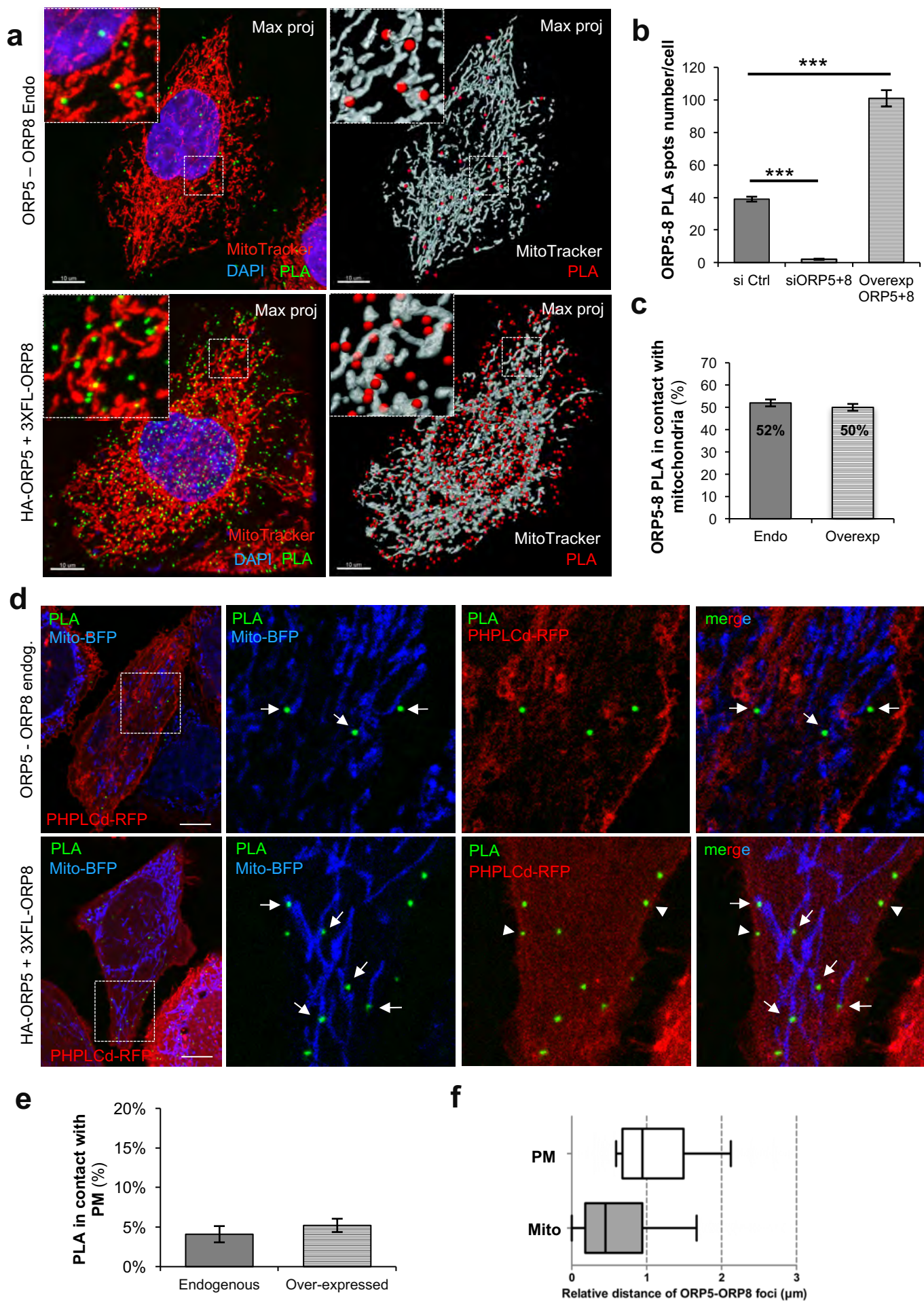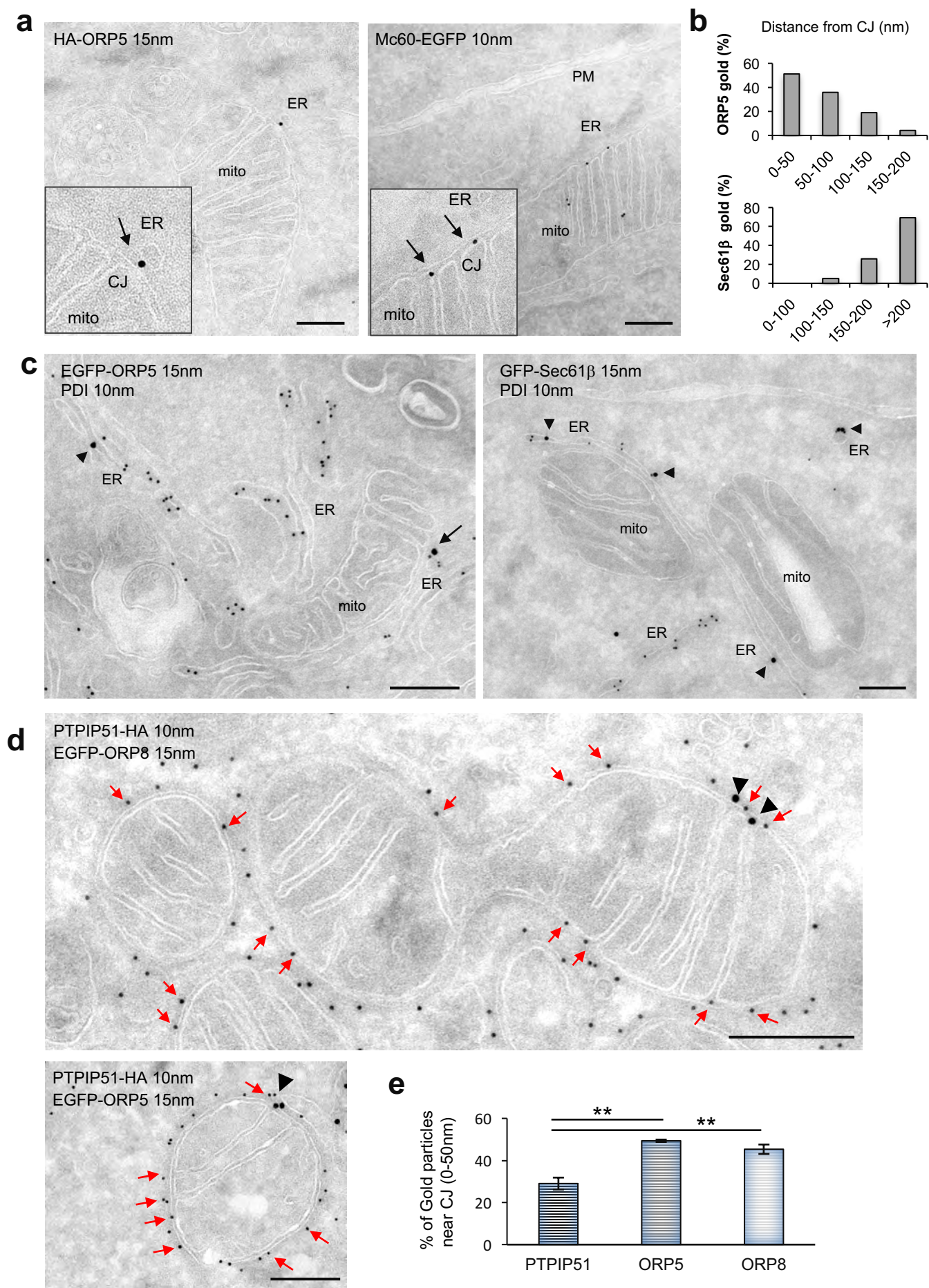1517

Figure 1

Figure 2

a

HA-ORP5 15nm

ER
mito
ER
CJ
mito

Mc60-EGFP 10nm

PM
ER
ER
CJ
mito
mito

b

Distance from CJ (nm)

ORP5 gold (%)

0-50   50-100   100-150   150-200

Sec61β gold (%)

0-100   100-150   150-200   >200

c

EGFP-ORP5 15nm
PDI 10nm

ER
ER
mito
ER

GFP-Sec61β 15nm
PDI 10nm

ER
mito
mito
ER
ER

d

PTPIP51-HA 10nm
EGFP-ORP8 15nm

PTPIP51-HA 10nm
EGFP-ORP5 15nm

e

% of Gold particles near CJ (0-50nm)

**   **

PTPIP51   ORP5   ORP8

Figure 3

a Mass-Spectrometry Analysis

Protein	Localization	IP EGFP	IP EGFP-ORP5	IP EGFP-ORP5ΔPH
			Mascot Score	
ORP5	MAMs	0	9689	12192
ORP8	MAMs	31	930	690
Mic60	IMM	43	579	1150
SAM50	OMM	0	237	516
Metaxin2	OMM	0	32	175
RHOT2	OMM	0	131	119
TOM22	OMM	0	77	95
PSS1	MAMs	0	150	200
PTPIP51	OMM	0	76	73

Figure 4

a

PLA ORP5-8 / Mitotracker — siCtrl | siSAM50 | siMic60

b

ORP5-ORP8 PLA to MAM (%)

***

siCtrl | siSAM50 | siMic60

c

siCtrl ORP5 Mitotracker / DAPI | siSAM50 ORP5 Mitotracker / DAPI

d

siCtrl ORP8 Mitotracker / DAPI | siSAM50 ORP8 Mitotracker / DAPI

e

ORP5 distribution

siCtrl
siSAM50

Distance to Mitochondria (µm)

ORP5 (%)

siCtrl | siSAM50

*** 0 µm
*** <0.38 µm
*** <0.5 µm

Distance to Mitochondria

f

ORP8 distribution

siCtrl
siSAM50

Distance to Mitochondria (µm)

ORP8 (%)

sictrl | siSAM50

*** 0 µm
*** <0.38 µm
*** <0.5 µm

Distance to Mitochondria

g

siCtrl | siSAM50 | siMic60

h

ER-mitochondria contacts

ER-mitochondria contacts surface extent (%)

n.s.

siCtrl | siMic60 | siSAM50

Figure 5

Figure 6

Figure 7

a

EGFP-ORP5	Anti-ORP5	merge
EGFP-ORP8	Anti-ORP8	merge
EGFP-ORP5	Anti-ORP5 HPA058727	merge

b

Pearson's correlation coefficient

- Anti-ORP5 vs ORP5: 0,704
- Anti-ORP8 vs ORP8: 0,81
- Anti-ORP5 HPA058727 vs ORP5: 0.80

c

αORP5 | αGAPDH

siCtrl, siORP5, siORP8, Marker | siCtrl, siORP5, siORP8, Marker

250 kDa
130 kDa
100 kDa
70 kDa
55 kDa
35 kDa
25 kDa
15 kDa

d

αORP8 | αGAPDH

Marker, siCtrl, siORP5, siORP8 | Marker, siCtrl, siORP5, siORP8

250 kDa
130 kDa
100 kDa
70 kDa
55 kDa
35 kDa
25 kDa
15 kDa

e

αORP5 HPA058727 | αGAPDH

Marker, siCtrl, siORP5 | Marker, siCtrl, siORP5 | Marker

250 kDa
130 kDa
100 kDa
70 kDa
55 kDa
35 kDa
25 kDa
15 kDa

f

Immunofluorecence

Fluorescence Intensity (%)

siCTRl αORP5 / siORP5 αORP5: -77%
siCTRl αORP8 / siORP8 αORP8: -57%

Western Blot

ORP5-8 / β-actin (%)

siCTRl αORP5 / siORP5 αORP5: -88%
siCTRl αORP8 / siORP8 αORP8: -65%

Figure S1

a

b

Figure S2

a

	PLA ORP5-8 endog	MitoTracker	merge	DAPI

siCtrl
Max proj / Max proj / Max proj

siORP5+8
Max proj / Max proj / Max proj

b

Confocal series > Objects detection > 3D distance map > Distance based object classification

PLA distance to mitochondria

3 µm

PLA distance to plasma membrane

10 µm

c

PLA ORP5-8 end	EGFP-Sec22b	Mito-BFP	merge	merge	merge

Figure S3

a

EGFP — PH — ORD — TM   ORP5

EGFP — PH — ORD — TM   ORP8

EGFP — ORD — TM   ORP5ΔPH

EGFP — ORD — TM   ORP8ΔPH

EGFP — PH — TM   ORP5ΔORD

EGFP — PH — ORD   ORP5ΔTM

b

Input | IP GFP

anti-GFP
← ORP5/ORP8
← GFP
anti-SAM50

c

EGFP-ORP5 | anti-SAM50 | MitoTracker | Merge

EGFP-ORP5ΔPH | anti-SAM50 | MitoTracker | Merge

d

GFP
Mic60
SAM50
Actin

e

$2^{e(-\Delta\Delta CT)}$

siCtrl
siSAM50

ORP5   SAM50   Mic60

**

****

Figure S4

Figure S5

a

Figure S6

a

ORD ORP5   ORD ORP8

b

Streptavidin-coated bead

Donor liposomes
Biotinylated, TopFluor-labeled

Acceptor liposomes

+/-
ORD

Fluorescent lipid transfert

c

Lipid transfer (%)

***

PS   +ORD8   PC   +ORD8   PE   +ORD8

Lipid transfer (%)

***

PS   +ORD5   PC   +ORD5   PE   +ORD5

d

Fluorescence intensity acceptor liposomes

Before float-up

Buffer   ORP8   ORP5   Buffer   ORP8   ORP5
PS   PC

Fluorescence intensity acceptor liposomes

After float-up

Buffer   ORP8   ORP5   Buffer   ORP8   ORP5
PS   PC

Figure S7

Figure S8

Figure S9