1 **Dynamic oil body generation in the marine oleaginous diatom *Fistulifera solaris***
2 **in response to nutrient limitation as revealed by morphological and lipidomic**
3 **analysis**

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21 **Abstract**

22 Microalgal lipids are promising useful natural compounds. Microalgae naturally
23 accumulate and store lipids in a subcellular organelle known as an oil body.
24 Understanding the cellular changes occurring during oil body generation is important
25 for unravelling the lipid accumulation mechanisms, variation in lipid homeostasis, and
26 further biotechnological application. A marine oleaginous diatom *Fistulifera solaris*
27 JPCC DA0580 is a potential candidate for biodiesel and eicosapentaenoic acid (EPA)
28 production. In this study, the lipidomic variations in *F. solaris* as well as the
29 morphological changes of oil bodies and chloroplasts were monitored following
30 nutrition-depletion. We observed that increases in oil body volume and decreases of
31 chloroplast volume accompanied the increase of neutral storage lipids and the
32 decrease of polar membrane lipids. Changes in chloroplast membrane lipids were
33 coordinated and differed from changes in extraplastidic membrane lipids. The large
34 decrease in polar lipids was concomitant with the significant decrease in newly
35 synthesized EPA-containing chloroplast membrane lipids, although EPA synthesis
36 was not significantly retarded. These findings suggest that *F. solaris* may selectively
37 shed EPA from chloroplast membrane lipids to generate triacylglycerols (TAGs) after
38 nutrition-depletion. We propose that this remodulation of lipid composition may
39 underlie the observed decrease in chloroplast volume.

40

41 **Keywords:** oleaginous diatom, oil body, chloroplast, membrane lipids remodeling,
42 eicosapentaenoic acid, nutrition-depletion

1. Introduction

Microalgae have long been known as a promising feedstock for the production of fatty acids-based useful compounds for food, chemical, pharmaceutical, and cosmetic industries [1, 2]. Polyunsaturated fatty acids (PUFA), especially n-3 PUFA, are valuable healthy supplement which have been demonstrated to be beneficial to various diseases [4]. A number of eukaryotic microalgal species naturally accumulate large amounts of lipids, mainly in the form of triacylglycerol (TAG), in a specialized cellular organelle known as oil body or lipid droplet [6]. Therefore, the oleaginous microalgae have also been recognized as possible candidates for biodiesel production [3].

Considerable reports have revealed that TAG accumulation in microalgae can be significantly enhanced or accelerated when cell growth was arrested by environmental stresses such as nutrient limitation [7-9]. TAG accumulation will more importantly change lipid and fatty acid composition and therefore affect the cellular lipid homeostasis [10-14]. Most lipidomic analyses with microalgae have demonstrated a decrease in polar lipids including chloroplast membrane lipids during TAG accumulation [7, 8, 12, 20, 22]. It has been proposed that the decrease in chloroplast membrane lipids may be used for the concomitant synthesis of TAGs. However, the dynamic changes in the cellular lipidome and its relation to the morphological changes of oil bodies and chloroplasts are still unclear. Moreover, the valuable PUFA have been reported to be present in high percentage in polar membrane lipids [7, 8, 11, 23]. Thus the accumulation of TAG would affect the content of PUFAs and/or their cellular distribution, the mechanism of which remains to be elucidated.

The marine oleaginous diatom *Fistulifera solaris* strain JPCC DA0580 was first identified by our group as a promising candidate for the production of microalgal fatty

68 acid-based valuable products, especially EPA and biodiesel, because of its high lipid
69 content and rapid growth [24, 25]. A unique feature of *F. solaris* is that there are
70 always two oil bodies per cell, together with a single chloroplast. In other words, lipid
71 accumulation only increases the volume of the two oil bodies and does not increase
72 the number of oil bodies. This feature facilitates morphological analyses such as
73 volumetric measurements of oil bodies and chloroplasts.

74 Our previous reports revealed that under nutrient rich condition, EPA were
75 concentrated in polar membrane lipids, whereas under nutrient limitation, with
76 moderate variation on cellular total EPA content, the EPA in polar lipids decreased
77 and the EPA in neutral lipids increased [11]. In addition, after 108 h of cultivation
78 under nutrient-limiting conditions, *F. solaris* cells can change their fatty acid
79 composition and in particular can accumulate significant amounts of neutral lipids [6,
80 11, 26]. However, the detailed temporal lipidomic variations and lipid homeostasis as
81 well as the related cellular physiological and morphological changes following
82 nutrient limitation is still unclear. In this study, we monitored the morphological and
83 lipidomic changes over time in nutrient-limiting conditions by applying previously
84 developed microcavity array-based single cell imaging system [27] and
85 UPLC-TOF-MS analysis. Cell growth and neutral lipid accumulation were also
86 monitored. The results reveal a coordinated series of changes in lipid content that are
87 concordant with the observed morphological changes and overall increase in TAGs.
88 Moreover, the redirection of EPA flow from chloroplast to oil body revealed in this
89 study may underlie the morphological change of chloroplasts.

90

91 **2. Materials and methods**

92 *2.1. Materials and instrumentation*

93 A CAPCELL PAK C18 IF (2 mm id $\times$ 50 mm, 2 μ m, Shiseido, Tokyo, Japan) was
94 used for the UPLC-TOF-MS analysis. The internal standard mixture (Human
95 Metabolome Technologies Inc., Yamagata, Japan) was used for the normalization of
96 peak detection information in UPLC-TOF-MS analysis. Methanol (Kanto Chemical,
97 Toyko Japan), acetonitrile (Kanto Chemical, Tokyo, Japan) and formic acid (Wako,
98 Osaka, Japan) were LC-MS grade. Chloroform (Wako, Osaka, Japan) and 2-propanol
99 (Kanto Chemical, Tokyo, Japan) were HPLC grade. All other reagents were of the
100 highest commercial grades available.

101 The UPLC-TOF-MS analyses were carried out using an Agilent 1200 series
102 Rapid Resolution LC (RRLC) system SL equipped with an Agilent G1969A LC/MSD
103 TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany). These systems
104 were controlled by Agilent G2201AA ChemStation software version B.03.01
105 (Agilent Technologies) for LC-MS operation and MasterHands (provided by Keio
106 University, Tsuruoka, Japan) for data analyses.

107

108 2.2. Cell culture and sampling

109 *F. solaris* was cultured using the two-phase cultivation strategy described
110 previously [6, 11, 26]. The nutrient-rich 10f medium which contained 10-fold more
111 nutrition components than f medium (150 mg NaNO₃, 12 mg Na₂HPO₄•2H₂O, 1 μ g
112 vitamin B12, 1 μ g biotin, 200 μ g thiamine HCl, 20 mg Na₂SiO₃•9H₂O, 8.8 mg
113 Na₂EDTA, 6.32 mg FeCl₃•6H₂O, 24 μ g CoSO₄•5H₂O, 42 μ g ZnSO₄•7H₂O, 0.36 mg
114 MnCl₂•4H₂O, 140 μ g CuSO₄•5H₂O, and 14 μ g Na₂MoO₄•2H₂O per liter of artificial
115 seawater (ASW)) [28] was selected to accelerate biomass production and to reduce
116 the intensive neutral lipid accumulation [11] during the first phase. After 72 h of
117 cultivation in 10f medium, cells were collected and transferred into ASW without any

118 additional nutrients to induce lipid accumulation. The initial cell concentration was
119 1.0×10^6 cells·mL⁻¹ and cells were cultured in flat-shaped flasks (1.5 L). Before
120 switching to ASW, 25 ml of culture in 10f medium was collected and lyophilized.
121 These cells were used as the sample for 0 h. After switching to ASW, 25 ml of culture
122 at 24 h, 60 h and 108 h were collected and lyophilized. These samples were prepared
123 for the lipidomics analysis. For confocal imaging and measurement of cellular
124 organelle volume, 1 ml of culture was collected every 24 hours after switching into
125 ASW and stained with BODIPY 505/515
126 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; Molecular Probes,
127 Eugene, OR, USA). Another three sets from two-phase cultivation were prepared for
128 the evaluation of neutral lipid content and cell growth. 25 ml of culture was collected
129 and harvested at 0 h, 12 h, 24 h, 36 h, 48 h, 60 h, 84 h, and 108 h after switching into
130 ASW. Cell concentrations were measured by direct counts using a microscope (Nikon
131 E200, Nikon, Tokyo, Japan). The temperature (26°C), light intensity (200
132 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), CO₂ concentration (2%), and the air flow rate (0.8 L/min) were as
133 described previously [6, 11, 26].

134

135 2.3. Confocal imaging to measure the volume of the intracellular organelles

136 Microalgal cells stained with BODIPY 505/515 were patterned in the agarose-gel
137 pad as previously described [27]. Briefly, cells were captured on microcavity arrays
138 [29] containing 102,400 cavities by applying negative pressure for 1 min from the
139 backside of the array. Following cell entrapment, an agarose solution was added onto
140 the microcavity array to fix the cells. The cells in the agarose-gel pad were then
141 observed under a confocal microscope (FV1000, Olympus Corp., Tokyo, Japan). The
142 z-stack images of the patterned cells were obtained with a 100 oil-immersion

143 objective. Three-dimensional reconstructions of confocal images were generated
144 using Volocity 5.0 image analysis software (Perkin Elmer, Waltham, MA, USA), and
145 fluorescent signal volumes were measured using intensity-based thresholds. The oil
146 body volume was determined by the fluorescent signal of BODIPY, and chloroplast
147 volume was determined by the signal of its autofluorescence.

148

149 *2.4. Lipid extraction*

150 The neutral lipids were extracted from the freeze dried cells with *n*-hexane and
151 dried under Ar gas. The neutral lipids were weighed, and the neutral lipid content was
152 expressed as a percentage of dried cell weight (DCW, % of DCW).

153 For lipidomic analysis, the total lipids were extracted from the lyophilized cells
154 of each sample using the two-step extraction methods described previously with
155 minor modifications [11, 26]. The solvent for the first-step extraction was mixed with
156 1/50 (v/v) of internal standard. Three rounds of extractions (5 mg of lyophilized cells
157 for a single extraction) were performed for each sample. The crude lipid extracts were
158 dried and dissolved in 1 ml of iso-propanol (IPA) and stored at -30°C before analysis.

159

160 *2.5. Lipidomic analysis using UPLC-TOF-MS*

161 Lipidomic analysis was carried out by UPLC-TOF-MS, equipped with a
162 reversed-phase column (CAPCELL PAK IF (2 mm id × 50 mm, 2 μm)). The gradient
163 elution buffers were A (H₂O containing 0.1% (v/v) formic acid) and B (isopropanol/
164 acetonitrile/ H₂O 65:30:5 (v/v/v) containing 0.1% (v/v) formic acid and 2 mM
165 ammonium formic acid). The flow rate was set at 0.3 ml/min and the column was
166 incubated at 40°C. The gradient elution was performed for 0–0.5 min 0%, 0.5–13.5
167 min 0% to 100%, and 13.5–20 min 100% of buffer B. The settings for MS were as

168 follows: ESI-TOF-MS was operated in the positive ion mode (capillary voltage: 4 kV)
169 for the analysis of DAG, TAG, MGDG, DGDG and PC, and the negative ion mode
170 (capillary voltage: 3.5 kV) for the analysis of SQDG and PG. The flow rate of heated
171 dry N₂ gas (heater temperature 350°C) was maintained at 40 psi. Precise mass data
172 were acquired over a range of 100-1700 m/z. The DAGs, TAGs, MGDGs and DGDGs
173 were detected by the [M+NH₄]⁺ ions, PCs were detected by [M+H]⁺ ions, and SQDGs
174 and PGs were detected by [M-H]⁻ ions. Each extract was analyzed three times.
175 Samples for the analyses at positive ion mode were diluted 20 times while the samples
176 for the analyses at negative ion mode were at the original concentration.

177

178 *2.6. Data processing and analyses*

179 Peak extraction was carried out with KEIO MasterHands software [30]. The
180 alignment of peaks was performed according to the m/z value and normalized
181 retention time based on the standards with corresponding structure. Peak areas were
182 normalized against those of the internal standards. The resultant relative area values
183 were further normalized by sample amount (weight (mg) of dried biomass) and
184 average values were calculated in each group. Due to the absence of the proper
185 standards for SQDG, the SQDGs were annotated according to the m/z value only.

186 The lipids were grouped into 6 clusters based on their 0 h based log fold changes
187 by the k-means clustering using the MultiExperiment Viewer 4.9 (MeV4.9;
188 <http://www.tm4.org/mev.html>) with Euclidean distance. Hierarchical trees were also
189 generated. Each of the clusters exhibits a particular pattern of temporal dynamics.

190

191 **3. Results and Discussion**

192 *3.1. Cell growth characteristics during nutrient limitation*

193 To relate lipid accumulation during nutrient limitation with the general physiology
194 of the cell, we first examined general growth characteristics. Cell concentrations
195 gradually increased during the first 24 h of nutrient limitation but then began to be
196 retarded (Fig. 1a). On the other hand, neutral lipid content steadily increased from
197 nearly 10% of dried cell weight (DCW) to 50% DCW during the same period of time
198 (Fig. 1a). The lipid accumulation rate in the first 24 h was slower than afterwards.
199 Based on these observations, we divided the 108 h period of cultivation under
200 nutrition-depletion into a cell division phase (0 h to 24 h) and a lipid accumulation
201 phase (24 h to 108 h). During this period we observed three different phases of neutral
202 lipid accumulation. The lowest lipid accumulation rate was from 0 h to 24 h and thus
203 may be influenced negatively by the higher cell division rates during this time. During
204 24 h to 48 h, lipid accumulation rates more than doubled and then steadily decreased
205 during the remainder of the time course. The reduced cell growth rates during this
206 time may result in more ATP and NADPH being available for TAG synthesis[31].

207

208 *3.2. Volumetric changes of oil bodies and chloroplasts are negatively correlated*

209 We then examined the alterations in oil body and chloroplast volume by
210 fluorescent microscopy, which revealed the concomitant increase in oil body size with
211 a decrease of chloroplast volume in response to nutrient limitation (Fig. 1b). For a
212 more precise evaluation of these opposing morphological changes, their volumes were
213 measured based on 3D images. We found that oil body volume steadily increased
214 from around 5 to almost 50 μm^3 during the 108 h time course (Fig. 1c). This
215 continuous increase in oil body volume is consistent with the changes in neutral lipid
216 content. However, while oil body volume increased more than 8-fold, neutral lipid
217 content increased just 6-fold during the same time period. This observation suggests

218 that not all the neutral compounds were extracted from the oil body by *n*-hexane.

219 On the other hand, chloroplast volume significantly decreased from about 40 μm^3
220 to less than 20 μm^3 during the time course (Fig. 1c). This decrease occurred during the
221 first 72 h, after which chloroplast volume was stable. More importantly, we found that
222 the increase in volume of the oil bodies (about 23 μm^3) was almost equivalent to the
223 decrease in chloroplast volume (about 24 μm^3) during the first 72 h. The reason for
224 this precise complementary relationship is still unclear, however, it suggested that
225 chloroplast membrane lipid turnover may be associated with neutral lipid
226 accumulation in response to nutrition limitation.

227

228 3.3. Cell division is associated with a significant decrease in polar lipids

229 To generate more detail about neutral lipid accumulation and membrane lipid
230 turnover, the temporal changes in lipids were examined by lipidomic analysis at
231 different times during the nutrient limitation time course. As sampling points we
232 chose 0 h, 24 h, 60 h and 108 h. During this period total TAGs increased steadily up
233 to 7-fold the starting levels (Fig. 2), reflecting closely the oil body volumetric change.
234 This result indicates that TAGs may be the principal compounds found in oil bodies.
235 However, the total DAGs slightly decreased at 24 h and then increased constantly to nearly
236 2.5-fold as compared to the 0 h level, which has not been expected before. Its decrease at the
237 first 24 h may attribute to the cell division while the increase after 24 h may be correlated
238 with the TAG accumulation after nutrient limitation because DAG is the direct precursor for
239 TAG synthesis. Moreover, DAG is also reported as a second messenger signaling lipid in
240 animal cell, its accumulation may also correlate with several cellular metabolic changes in
241 diatoms which we do not know. The lipid mediated signaling would be very important for
242 stress response of microalgae especially oleaginous microalgae and need further studies.

243 On the other hand, polar membrane lipids decreased during nutrient limitation.

244 The most drastic decrease in all polar lipid classes (including MGDG, DGDG, SQDG,
245 PC, and PG) was observed during the cell division phase, after which the changes
246 were moderate (Fig. 2). These findings suggest that cell division might be the reason
247 underlying both the drastic decrease of most polar lipid classes and the decrease of
248 chloroplast volume during the same time period. The massive accumulation of TAGs
249 in response to nutrient limitation may therefore be attributable to *de novo* synthesis
250 through the classic Kennedy pathway [32] rather than membrane lipid turnover.
251 Although the volume of chloroplasts halved after nutrient starvation, the integrity of
252 chloroplast as well as the thylakoid structure are maintained (Supplementary Figure 1).
253 The same phenomenon has been reported to be found in *Nannochloropsis gaditana*
254 [7]. The shrunken chloroplasts could still perform effective photosynthesis, even
255 though the photosynthetic efficiency were decreasing following nutrient starvation.
256 Previously, Burrows et al. demonstrated that the TAG in *Phaeodactylum tricornutum*
257 was mainly *de novo* synthesized from photosynthetic CO₂ fixation after nitrogen
258 limitation rather than the turnover of initial pool [33]. Therefore, the precursors for
259 fatty acids synthesis in *F. solaris* after nutrient limitation may also mainly derived
260 from photosynthetic carbon fixation.

261

262 3.4. Chloroplast membrane lipids show complex variations The results above
263 revealed that while chloroplast volume decreased continuously up to 72 h (Fig. 1c),
264 the total content of MGDG, DGDG, SQDG and PG, which are recognized as common
265 chloroplast membrane lipids, displayed only moderate changes after the cell division
266 phase (Fig. 2). To understand this discrepancy further, more detailed analyses of the
267 lipidomic data were performed. A previous report revealed that PC represents
268 approximately 15% of thylakoid membrane lipids [36]. However, the exact

269 localization of PC on the thylakoid membrane and the impact of their variation to the
270 chloroplast volume change are vague and would not be discussed here. A total of 167
271 LC-MS peaks detected by UPLC-TOF-MS analysis were assigned as possible lipid
272 molecules. The detailed composition of the predicted lipid molecules in each lipid
273 class and their variation after nutrient depletion are summarized in Supplementary
274 Figures 2-9. Two culture replicates and three analysis replicates generated identical
275 results. The changes of each individual lipid species following nutrient depletion
276 could be resolved into six clusters based on their 0 h-based log 2 fold changes (Fig.
277 3).

278 Lipids in clusters 1 and 2 were mostly TAGs and continuously increased during
279 nutrient limitation. The rate of increase of abundance of the lipids grouped in cluster 1,
280 mainly the major TAG molecules (Supplementary Fig. 2 and [11]), was much higher
281 than that of the lipids grouped in cluster 2. Most of the polar lipids were grouped in
282 the other clusters, and decreased over the time course. These results are consistent
283 with the overall lipid changes and the morphological changes of oil bodies and
284 chloroplasts.

285 One surprising result was that cluster 3, which contains exclusively the common
286 chloroplast membrane lipids, showed a continuous decrease in response to nutrient
287 limitation, while cluster 6, containing the PC, initially decreased and then increased
288 back to their initial levels. These findings suggest that the concentrations of
289 chloroplast membrane lipids and extraplastidic membrane lipids are differentially
290 controlled in response to nutrient depletion. The chloroplast membrane lipids in
291 cluster 3 may be related to the decrease of chloroplast volume after cell division.

292

293 *3.5. Newly synthesized EPA-containing chloroplast membrane lipids and highly*

294 *unsaturated C32 chloroplast membrane lipids are selectively and rapidly decreased*

295 The log-fold changes observed in major lipid species at different times of
296 nutrient limitation with respect to the time zero control have been organized onto the
297 putative lipid synthesis pathways of *F. solaris* [39] in Figure 4. The diatom chloroplast
298 is covered by four membranes rather than two [40]. The four envelope membranes are
299 the innermost membrane, the second innermost membrane, the second outermost
300 membrane and the outermost membrane from inside to outside. The space between
301 the innermost and second innermost membrane can be recognized as the primary
302 chloroplast. The space between the second outermost membrane and the outermost
303 membrane is defined as chloroplast endoplasmic reticulum (CER) which may
304 preserve the lipid composition and more importantly some function of the normal ER.
305 We propose that as being similar to plants and green algae, MGDG, SQDG, DGDG,
306 and PG are synthesized in the inner chloroplast, while PC is synthesized in the CER
307 or ER. The lipids were selected based on the percentage distribution in each lipid
308 classes (Supplementary Fig. 3-9) and their fatty acid composition were determined by
309 direct infusion ESI-Q-TOF-MS/MS analysis.

310 Based on our previous study [11, 26], due to the extremely low content of C18
311 fatty acids in glycolipids, the chloroplast membrane lipids could be roughly divided
312 into 2 groups, the EPA-containing lipids (C36) and non-EPA containing lipids (C32
313 and C30) . We further demonstrated that the non-EPA containing lipids were
314 exclusively synthesized in the chloroplast [39] while the EPA was synthesized in the
315 ER or CER and then transported into the chloroplast for the synthesis of
316 EPA-containing lipids in the chloroplast [26, 41]. Although the lipid trafficking
317 between ER (or CER) and inner chloroplast in diatom is still unclear, it may share
318 some similar mechanisms with that in higher plants and green algae [42, 43]. Since

the C16:2 and C16:3 are known to be desaturated at the acyl-lipid level in the chloroplast [41], we also divided the chloroplast membrane lipids into the newly synthesized lipids and highly desaturated lipids based on the unsaturation level of the C16 fatty acids.

As mentioned above, the polar lipids showing the most dramatic reductions were present in cluster 3 (Fig. 3). Interestingly, these lipids included newly synthesized EPA-containing MGDG, DGDG and PG, as well as the highly unsaturated C32 MGDG (Fig. 4). On the other hand, the newly synthesized C32 MGDG and SQDG, which grouped in cluster 4, were only moderately affected. The newly synthesized C30 SQDG and DGDG, and C32 DGDG, which were grouped into cluster 5, were essentially unchanged both during and after the cell division phase. These findings indicated that the loss of the newly synthesized EPA-containing and highly unsaturated C32 MGDG chloroplast membrane lipids may be the main force driving the chloroplast size reduction during nutrient limitation.

The dramatic and specific decrease of newly synthesized EPA-containing chloroplast membrane lipids also suggested an altered EPA-flow in response to nutrient limitation. As shown in Supplementary Figure 3, the percentage and actual amounts of EPA-containing TAGs increased during the time course, similar to what has been reported in *Nannochloropsis* sp. [44]. A study on *P. tricornutum* membrane remodeling during nitrogen and phosphorus triggered TAG accumulation also showed a decrease in total EPA-containing MGDGs and an increase in total EPA-containing TAGs [8]. On the other hand, the C18-containing PCs, which have been demonstrated to be the intermediates for EPA synthesis in *F. solaris* [26, 41], were only moderately affected by nutrient limitation (Fig. 4 and Fig. 3 cluster 5). These results strongly suggest that the observed decrease in newly synthesized EPA-containing chloroplast

344 membrane lipids was not due to the downregulation in EPA synthesis. The specific
345 decrease in newly synthesized EPA-containing chloroplast membrane lipids could
346 thus be due to the selective activation of specific lipases or the blocked EPA transport
347 from ER or CER to chloroplast rather than a reduced EPA supply.

348 A previous study in the green alga *Chlamydomonas reinhardtii* revealed that a
349 galactosyl lipid lipase, which selectively degraded the newly synthesized MGDG, was
350 involved in TAG accumulation [31]. It is possible therefore that a similar lipase in *F.*
351 *solaris* can specifically degrade the newly synthesized C36 MGDG and PG. The
352 mechanism for transporting the resulted EPA-CoA from inner chloroplast to CER or
353 ER for the TAG synthesis is unclear. This process is probably occurred around the
354 membrane contact sites (MCSs) [43]. We propose that chloroplast membrane turnover,
355 especially the decrease of the newly synthesized EPA-containing chloroplast
356 membrane lipids, may contribute to the increase of EPA-containing TAGs. These
357 EPA-containing chloroplast membrane lipids may also be essential for the
358 maintenance of chloroplast membrane structure. Thus, the rerouting of EPA from the
359 chloroplast membrane to the oil body during nutrient limitation may underlie the
360 reduction in chloroplast size, especially after the cell division phase.

361 We summarized the major observations in this study into a schematic figure to
362 illustrate the lipidomic variations underlying the specific cell morphological changes
363 in *F. solaris* in response to nutrient limitation (Fig. 5). *F. solaris* cells tend to
364 accumulate TAG and enlarge the oil bodies in response to nutrient limitation.
365 Meanwhile chloroplast volume decreases significantly, which we propose to be
366 attributed to the redirectioning of EPA from chloroplast membrane lipids into TAG.
367 Normally, EPAs are synthesized in the ER or CER and are then incorporated into
368 glycerolipids when nutrients are in surplus. However, in nutrient limited conditions,

369 even though EPA synthesis was largely unaffected, the newly synthesized
370 EPA-containing chloroplast membrane lipids decreased dramatically. This decrease
371 may be due to the activation of a specific lipase or the blocking of EPA transport to
372 chloroplasts and the continuous desaturation of these lipids. Although the exact
373 mechanism for lipid trafficking through the four chloroplast membranes is still
374 unclear, the EPA recycled from the inner chloroplast may be transported to CER or
375 ER for TAG synthesis through the MCSs. On the other hand, the desaturation of
376 newly synthesized C32 MGDGs may be retarded, which would result in the
377 significant reduction of the highly unsaturated C32 MGDGs. Our observations
378 strongly suggest that the newly synthesized EPA-containing chloroplast membrane
379 lipids and the highly unsaturated C32 MGDGs are important for the maintenance of
380 chloroplast membrane integrity.

381

382 **4. Conclusion**

383 The morphological and lipidomic analyses reported here in the marine
384 oleaginous diatom *Fistulifera solaris* reveal a negative correlation between oil body
385 size increases and chloroplast size reductions, and suggest that TAG accumulation in
386 response to nutrient limitation was driven principally by *de novo* synthesis through the
387 Kennedy pathway. Our data also reveal that EPA synthesis is largely unaffected by
388 nutrient limitation but is likely a consequence of the redirectioning of EPA from
389 chloroplast membrane lipids to TAG, which we propose to be responsible for the
390 decrease of chloroplast volume. These findings may facilitate the further
391 understanding of lipid accumulation in microalgae and the genetic engineering of *F.*
392 *solaris* towards the production of biodiesel or EPA.

393

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521 Figure 1. Cell growth characterization, neutral lipids variation and volumetric changes
522 of oil bodies and chloroplasts in *F. solaris* in response to nutrient limitation. a) growth
523 curve (solid line) and variation of neutral lipid content (dashed line) in *F. solaris* in
524 response to nutrient limitation. Error bars indicate $\pm$ SD. (n=3, analyses of 3
525 independent cultures). b) representative confocal fluorescent microscopy images of *F.*
526 *solaris* following nutrient limitation. Red fluorescence, chloroplast autofluorescence;
527 green fluorescence, oil bodies stained with BODIPY 505/515. c) volumetric changes
528 of oil bodies (solid line) and chloroplasts (dashed line) following nutrient limitation.
529 Error bars indicate $\pm$ SE. (n=30, analyses of 10 cells from each of 3 independent
530 cultures).
531

a)

b)

Scale bar = 5 μ m

c)

533

534

537 Figure 2 Changes in the content of different lipid classes in *F. solaris* strain JPCC
 538 DA0580 in response to nutrient limitation. Error bars indicate $\pm$ SD. (n=6, 3
 539 independent analyses of lipid extracts from 2 independent cultures).

538

539

539 Figure 3 Lipidomic dynamics of 84 lipid species in response to nutrient limitation.
540 Variations in patterns of the different lipids could be grouped into 6 clusters. Mean log
541 fold changes of lipids were sorted as a green-black-red gradient. The numbers before
542 the colons indicate the total carbon atoms in the fatty acid chain and the numbers after
543 the colons mean the numbers of total double bonds between adjacent carbon atoms in
544 the structure. Note that lipid species that were not detectable at one of the time points
545 were not included in the clustering analysis.

Cluster 1

Cluster 2

Cluster 3

Cluster 4

Cluster 5

Cluster 6

547

548

553 Figure 5 Schematic figure showing the proposed flux of EPA (C20:5) from
554 chloroplast membrane lipids and newly synthesized EPA to TAG in response to
555 nutrient limitation. In nutrition-replete conditions (A), the synthesized EPAs are
556 incorporated into TAG, PC and chloroplast membrane lipids (MGDG, DGDG, SQDG,
557 and PG). In nutrient limiting conditions (B), the flux of EPA to chloroplast membrane
558 lipids might be down-regulated by several unknown mechanisms (x-marked arrows),
559 thus leading to an increase in EPA-containing TAGs (red arrows). Pairs of numbers
560 representing fatty acids, when separated by a slash, represent the components in the
561 *sn*-1 and *sn*-2 positions, respectively, of the molecular species.

562

A) Nutrition +

B) Nutrition -

564