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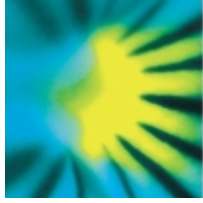
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Long-chain acyl-CoA synthetases activate fatty acids for lipid synthesis, remodeling and energy production in *Chlamydomonas*

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1 **Long-chain acyl-CoA synthetases activate fatty acids for lipid**
2 **synthesis, remodeling and energy production in Chlamydomonas**

3

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12

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14

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20 supplementary table, and 2 data sets.

21 **Summary**

- 22 • Long-chain acyl-CoA synthetases (LACSs) play many roles in mammals, yeasts
23 and plants, but knowledge on their functions in microalgae remains fragmented.
24 Here via genetic, biochemical and physiological analyses, we unraveled the
25 function and roles of LACSs in the model microalga *Chlamydomonas*
26 *reinhardtii*.
- 27 • *In vitro* enzymatic activity assays on purified recombinant proteins revealed that
28 CrLACS1, CrLACS2 and CrLACS3 all exhibited bona fide LACS activities
29 toward a broad range of free fatty acids.
- 30 • The *Chlamydomonas* mutants compromised in CrLACS1, CrLACS2 or
31 CrLACS3 didn't show obvious phenotypes in lipid content or growth under
32 nitrogen (N)-replete condition. But under N-deprivation, *CrLACS1* or *CrLACS2*
33 suppression resulted in ~50% less oil, yet with a higher amount of chloroplast
34 lipids. By contrast, *CrLACS3* suppression impaired oil remobilization and cell
35 growth severely during N-recovery, supporting its role in fatty acid β -oxidation
36 to provide energy and carbon sources for regrowth. Transcriptomics analysis
37 suggested that the observed lipid phenotypes are likely not due to transcriptional
38 reprogramming but rather a shift in metabolic adjustment.
- 39 • Taken together, this study provided solid experimental evidence for essential
40 roles of the three *Chlamydomonas* LACS enzymes in lipid synthesis,
41 remodeling and catabolism, and highlighted the importance of lipid homeostasis
42 in cell growth under nutrient fluctuations.

43

44 **Keywords**

45 Acyl activation, fatty acid β -oxidation, lipid droplet, lipid homeostasis, lipid
46 remodeling, N recovery, triacylglycerol

47 **Introduction**

48 Microalgae have emerged as a promising platform for production of food, fuels and
49 synthons for green chemistry (Tang *et al.*, 2016; Levasseur *et al.*, 2020). Many of the
50 potential applications are related to their capacity in synthesizing and accumulating a
51 wide range of fatty acid structures and the potential for high amount of neutral lipids
52 e.g. triacylglycerol (TAG) or oil (Hu *et al.*, 2008). Fatty acids are building blocks of
53 membrane and storage lipids and serve as important substrates for carbon and energy
54 storage, energy production, acylation of proteins and for lipid-based signaling (Glatz &
55 Luiken, 2015; Resh, 2016; De Carvalho & Caramujo, 2018). To fulfil above functions,
56 fatty acids need to be activated either to acyl carrier proteins (ACPs) in the chloroplast
57 or to Coenzyme A (CoA) in the extra-chloroplast compartments and the latter process
58 is mediated by the ATP-dependent long-chain acyl-CoA synthetases (LACSs) (Li-
59 Beisson *et al.*, 2015; Zhao *et al.*, 2021). Acyl-CoAs are important metabolic
60 intermediates in *de novo* acyl-lipid synthesis, in re-integration of free fatty acids (FFAs)
61 coming from membrane lipid degradation back to lipid metabolism and in mediating
62 fatty acid β -oxidation in peroxisomes, among others. Therefore, LACS enzymes play a
63 central role in acyl-lipid metabolism.

64 The functions and physiological roles of LACS family have been well studied in
65 many organisms including bacteria, yeasts, vascular plants and mammals (DiRusso &
66 Black, 1999; Coleman *et al.*, 2002; Shockey *et al.*, 2002; Zhao *et al.*, 2010; Watkins &
67 Ellis, 2012; Zhao *et al.*, 2019; Ayaz *et al.*, 2021) but rare in microalgae. For example,
68 *Escherichia coli* harbors a single LACS encoded by the *FadD* gene, which resides at
69 the inner membrane of cell envelope and converts FFAs to acyl-CoAs for downstream
70 lipid metabolism (Black *et al.*, 1992). It also functions in combination with the outer
71 membrane-bound fatty acid transport protein FadL to facilitate import of exogenous
72 FFAs (DiRusso & Black, 1999). On the other hand, there are four LACS isoforms
73 reported in *Saccharomyces cerevisiae*, namely, Faa1p through Faa4p (Johnson *et al.*,
74 1994; Knoll *et al.*, 1995). Whilst Faa1p, Faa3p and Faa4p are involved in activation of
75 FFAs to form acyl-CoAs for lipid synthesis (Færgeman *et al.*, 2001; Kamisaka *et al.*,

76 2007), Faa2p functions in peroxisomes for fatty acid β -oxidation (Hettema *et al.*, 1996).
77 Moreover, Faa1p or Faa4p cooperates with a plasma membrane-bound fatty acid
78 transporter, Fat1p, to facilitate import of exogenous FFAs (Zou *et al.*, 2003).

79 By contrast, there are many more LACS isoforms in vascular plants, e.g., nine in
80 Arabidopsis (Shockey *et al.*, 2002) and they are present in plastid, ER or peroxisomes
81 (Schnurr *et al.*, 2002; Fulda *et al.*, 2004; Zhao *et al.*, 2010). Despite all nine LACS
82 isoforms (LACS1 through LACS9) function in converting FFAs to acyl-CoAs *in vitro*
83 (Shockey *et al.*, 2002), their roles in Arabidopsis are multifaceted (Fulda *et al.*, 2002;
84 Schnurr *et al.*, 2002; Fulda *et al.*, 2004; Lü *et al.*, 2009; Weng *et al.*, 2010; Zhao *et al.*,
85 2010; Jessen *et al.*, 2011; Jessen *et al.*, 2015; Zhao *et al.*, 2019). It has been
86 demonstrated that the three ER LACS (LACS1, LACS2 and LACS4) are involved in
87 cuticular lipid metabolism in Arabidopsis (Lü *et al.*, 2009; Weng *et al.*, 2010; Jessen *et al.*,
88 2011). The peroxisomal LACS6 and LACS7, on the other hand, have overlapping
89 roles in fatty acid β -oxidation and are essential for seedling development (Fulda *et al.*,
90 2002; Fulda *et al.*, 2004). Albeit being of close phylogenetic relationship, LACS8 and
91 LACS9 are targeted to the ER and plastid outer envelope, respectively (Schnurr *et al.*,
92 2002; Zhao *et al.*, 2010). Interestingly, LACS9, but not LACS8, functionally overlaps
93 with LACS1 in seed TAG biosynthesis (Zhao *et al.*, 2010). Moreover, both LACS8 and
94 LACS9 have functional overlap with LACS4 in TAG synthesis in seeds (Jessen *et al.*,
95 2015; Zhao *et al.*, 2019). In this context, there is a complex network of functions and
96 interactions among LACS isoforms in Arabidopsis (Zhao *et al.*, 2019).

97 Only limited efforts have so far been made on studying LACSs from microalgae
98 and their functional roles *in vivo* remain largely unknown (Zhang *et al.*, 2012; Guo *et al.*
99 *et al.*, 2014; Jia *et al.*, 2016; Li *et al.*, 2016; Wu *et al.*, 2020). The green alga
100 *Chlamydomonas reinhardtii* (hereafter Chlamydomonas) has been widely used as a
101 unicellular model autotroph for studying lipid metabolism (Merchant *et al.*, 2012; Li-
102 Beisson *et al.*, 2015; Kong *et al.*, 2019). There are three putative LACS-coding genes
103 in Chlamydomonas genome, namely, *CrLACS1*, *CrLACS2* and *CrLACS3* (Li-Beisson
104 *et al.*, 2015). It has been reported that suppression of *CrLACS2* by insertional

105 mutagenesis impaired TAG accumulation under nitrogen (N)-deprivation (Li *et al.*,
106 2016). Knockdown of *CrLACS1* or *CrLACS3* via RNA interference was reported to
107 result in free fatty acid secretion (Jia *et al.*, 2016). Nevertheless, evidence is still lacking
108 with respect to how they function in lipid metabolism and their respective roles in lipid
109 homeostasis during *Chlamydomonas*' response to nutrient fluctuations.

110 In the present study, to reveal the function and physiological roles of CrLACS
111 members in *Chlamydomonas*, we conducted a multifaceted study by systematically
112 integrating *in vitro* LACS enzymatic activity assays, growth and lipidomic analyses of
113 insertional mutants and their corresponding complemented strains, and comparative
114 transcriptomics. All three LACSs were demonstrated as bona fide LACS enzymes and
115 showed activities toward a broad range of FFAs without apparent substrate preference.
116 Both CrLACS1 and CrLACS2 were involved in N-deprivation induced TAG synthesis,
117 with the former being more transcriptionally stimulated and likely playing a more
118 important role in TAG accumulation than the latter. Furthermore, CrLACS1 and
119 CrLACS2 were found to participate in directing acyl flux to TAG or chloroplast
120 membrane lipids. CrLACS3, on the other hand, was involved in TAG catabolism under
121 N-recovery to provide carbon precursors and energy for cell growth via the fatty acid
122 β -oxidation. We also discussed the possible spatial and temporal coordination of the
123 three CrLACS isoforms in maintaining lipid homeostasis in *Chlamydomonas* in
124 response to nutrient fluctuations.

125

126 **Materials and Methods**

127 **Algal strains and culture conditions**

128 The *Chlamydomonas* wild-type (WT) CC-5325 and insertional mutants including
129 *crlacs1-1* (LMJ.RY0402.173140), *crlacs1-2* (LMJ.RY0402.129310), *crlacs2-1*
130 (LMJ.SG0182.015843), and *crlacs3-1* (LMJ.RY0402.199483) were purchased from
131 the *Chlamydomonas* Resource Center. All strains were cultivated in Tris-Acetate-
132 Phosphate (TAP) medium under continuous illumination of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 150
133 rpm shaking at 23°C. For N-replete growth, cells were inoculated in TAP medium at a

134 cell density of 1×10^6 cells mL^{-1} and allowed to grow for 3 days. The algal cells were
135 then harvested by centrifuging, washed twice with N-free TAP medium (TAP-N) and
136 finally re-suspended in TAP-N at a cell density of 3×10^6 cells mL^{-1} for N-deprivation
137 treatment (ND). After 3 days of ND, cells were collected and equal volumes of TAP-N
138 were replaced by Tris-Minimal Medium (MM), followed by 3 days of growth in the
139 dark for N-recovery treatment (NDR).

140 Cell number and dry weight measurements were performed according to our
141 previously described procedures (Liu *et al.*, 2016a). For visualization of lipid droplets
142 (LDs), cells were stained with the fluorescent dye BODIPY 505/515 (Molecular Probes,
143 OR, USA) at a final concentration of $1 \mu\text{g mL}^{-1}$ for 10 min at room temperature, and
144 then observed under an Olympus BX51 fluorescence microscope (Olympus, Japan).

145

146 **RNA isolation and RT-qPCR**

147 Total RNA was isolated from algal samples (ca. 10^7 cells) using TRI reagent (Invitrogen,
148 Carlsbad, CA) according to the manufacturer's instructions. The cDNA synthesis and
149 RT-qPCR were performed following our previously described procedures (Liu *et al.*,
150 2016a). Primers used for RT-qPCR are listed in Table S1. The gene expression levels
151 were expressed relative to the internal control β -actin gene.

152

153 **Functional assessment of CrLACS1, CrLACS2 and CrLACS3 in yeast**

154 The coding sequences of *CrLACS*s were PCR amplified using primers listed in Table
155 S1 and sub-cloned into the yeast expression vector pYES2-CT (Invitrogen). The
156 resultant plasmids (Fig. S1a) were each transformed into the *S. cerevisiae* strain YB525
157 using S.c. EasyComp Transformation Kit (Invitrogen). YB525 is defective in the
158 LACS-coding genes *Faa1* and *Faa4* and thus cannot grow on FFAs as the sole carbon
159 source (Zou *et al.*, 2003). For growth test, YB525 cells expressing *CrLACS* genes were
160 first cultured in yeast synthetic medium lacking uracil (SD/-ura) containing 2% (w/v)
161 galactose for 4 h to induce *LACS* expression, followed by removal of galactose and
162 supplementation with FFAs at a concentration of $100 \mu\text{M}$ and incubation at 30°C for

163 ten days (Wu *et al.*, 2020). The FFA stock solutions were prepared in 10% isopropanol
164 containing 1% Triton-100. Yeast cell densities were recorded as OD₆₀₀.

165

166 **Purification of recombinant CrLACS1, CrLACS2 and CrLACS3 and *in vitro*** 167 **enzymatic activity assay**

168 The coding sequences of *CrLACS* genes were PCR amplified using primers listed in
169 Table S1 and sub-cloned into the *E. coli* expression vector pET32a. The resultant
170 plasmids (Fig. S1b) were each transformed into the *E. coli* strain BL21 (DE3), using
171 pET32a as the control. To induce protein expression, *E. coli* cells carrying *CrLACS*
172 genes were grown at 16°C for 16 h in the presence of 1 mM isopropyl-β-thiogalactoside
173 (IPTG). Then, *E. coli* samples were collected and lysed by sonication, followed by
174 centrifugation at 13,000 *g* for 15 min to remove cellular debris. The supernatant was
175 loaded onto a prepacked HisPur Ni-NTA column (ThermoFisher Scientific, Waltham,
176 MA, USA). To elute the target protein off the column, elution buffer that contains 200
177 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl buffer (pH 8.0) was applied and
178 the effluent was dialyzed against 100 mM Tris-HCl buffer (pH 8.0) to remove imidazole.
179 A 30 kDa cut-off concentrator (Millipore, Billerica, MA, USA) was used to concentrate
180 the target protein. The purified protein was subjected to SDS-PAGE analysis. The *in*
181 *vitro* activities of CrLACS isoforms were measured using an enzyme-coupled method
182 (Ichihara & Shibasaki, 1991) with a few modifications (Wu *et al.*, 2020). LACS
183 enzymatic activities were expressed as nmol acyl-CoA synthesized per min per mg
184 protein.

185

186 **Molecular characterization of insertional mutants of CrLACS1 and CrLACS3**

187 The mutants were streaked on TAP agar plates for colony formation. A single colony
188 from each mutant was then picked up and allowed to grow in liquid medium for
189 reproduction. To confirm the insertions, three PCR reactions using genomic DNA as a
190 template were conducted for each mutant, following the ChlaMmeSeq protocol (Zhang
191 *et al.*, 2014), which were (1) the predicted insertion site using the suggested primer pair

192 (<https://www.chlamylibrary.org>), (2) the 5' end of insertion cassette-genome junction,
193 and (3) the 3' end of insertion cassette-genome junction. Moreover, RT-qPCR was
194 employed to check the effect of insertions on transcriptional expression of *CrLACS*
195 genes. The primers were listed in Table S1.

196

197 **Functional complementation of mutants defected in CrLACS1 or CrLACS3**

198 The coding sequences of *CrLACS1* and *CrLACS2* were each sub-cloned into the
199 Chlamydomonas expression vector pOpt_Clover_Hyg (Lauersen *et al.*, 2015). The
200 resultant plasmids (Fig. S1c) were introduced into *crlacs1-1* and *crlacs3-1*, respectively,
201 for functional complementation, using the glass bead-mediated transformation method
202 (Kindle, 1990). The transformed cells, after overnight recovery under dim light, were
203 selected on TAP medium supplemented with 15 $\mu\text{g mL}^{-1}$ hygromycin under continuous
204 illumination of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Putative colonies were verified by genomic PCR and
205 RT-qPCR.

206

207 **Lipid extraction and analysis**

208 Lipids from yeast or Chlamydomonas were extracted with chloroform–methanol (2:1,
209 v/v) as previously described (Liu *et al.*, 2016a). To separate lipids by thin-layer
210 chromatography (TLC), lipids were loaded on silica gel 60 TLC plates (Merck,
211 Germany) and developed with a mixture of hexane/tert-butylmethyl ether/acetic acid
212 (80/20/2, by vol) for neutral lipids and with a mixture of chloroform/methanol/acetic
213 acid/water (25/4/0.7/0.3, by vol) for polar lipids (Liu *et al.*, 2016b). Individual lipids on
214 TLC plates were then stained with iodine vapor and recovered for subsequent
215 transesterification experiments.

216 Total lipids and recovered fractions of individual lipid classes were transesterified
217 to fatty acid methyl esters and then analyzed using an Agilent 7890 capillary gas
218 chromatograph equipped with a 5975C mass spectrometry detector and a HP-88
219 capillary column (60 m \times 0.25 mm) (Agilent Technologies, Santa Clara, CA), and
220 quantified according to our previously described procedures (Liu *et al.*, 2019).

221

222 RNA sequencing and differentially expressed gene analysis

223 WT, *crlacs1-1*, *crlacs2-1* and *crlacs3-1* under ND and NDR conditions were sampled
224 (biological duplicates) for RNA extraction. After treatment with RNase-free DNase I
225 (TaKaRa, Japan), total RNA (ca. 10 µg) from each sample was used for transcriptome
226 library construction and sequencing as detailed in our previous study (Shi *et al.*, 2020).
227 The clean reads were aligned to the genome of *C. reinhardtii* v5.5 in Phytozome v.12
228 (<https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.jsf?organism=P>
229 hytozome) with the software TopHat (version 2.0.4) (Trapnell *et al.*, 2012). Reads
230 mapping to more than one location were excluded. The transcriptome data were
231 submitted to the NCBI SRA database with the accession number PRJNA736801. The
232 gene transcriptional abundance was expressed as fragments per kilobase million
233 (FPKM). Differentially expressed genes (DEGs) between mutants and WT met the
234 following requirements: at least a 2-fold change with $P_{\text{adjust}} < 0.001$ and the FPKM
235 value of either sample > 1 .

236

237 Results**238 Chlamydomonas encodes three putative LACSs with distinct subcellular locations**

239 Chlamydomonas harbors three putative LACS isoforms, CrLACS1, CrLACS2 and
240 CrLACS3. To understand the evolution of CrLACSs, a phylogenetic tree was
241 constructed using the three LACS proteins and the homologs from other organisms (Fig.
242 S2). Apparently, CrLACS1 and CrLACS2 are closely related to *Chromochloris*
243 *zofingiensis* LACS2-4 and clustered with Arabidopsis LACS1, LACS4 and LACS5,
244 which are ER-localized (Zhao *et al.*, 2019; Wu *et al.*, 2020). Interestingly, proteomics
245 analysis of purified lipid droplets (LDs) revealed association of CrLACS2 (Moellering
246 & Benning, 2010; Nguyen *et al.*, 2011) and the *C. zofingiensis* CzLACS2-4 (Wang *et al.*
247 *et al.*, 2019) to LDs. This is reasonable as cytosolic LDs are believed to be derived from
248 the ER (Ischebeck *et al.*, 2020) and thus may contain ER proteins including LACSs.
249 Taken together, CrLACS1 and 2 are likely ER proteins and with CrLACS2 being

250 enriched in LD fraction.

251 CrLACS3, on the other hand, is closely related to *C. zofingiensis* LACS5 and
 252 clustered with *Arabidopsis* LACS6 and LACS7, known to be peroxisome-localized
 253 (Fulda *et al.*, 2004; Wu *et al.*, 2020). This, together with the fact that the
 254 *Chlamydomonas* fatty acid β -oxidation occurs in peroxisomes (Kong *et al.*, 2017),
 255 makes it highly likely that CrLACS3 is located in peroxisomes where it activates free
 256 fatty acids for β -oxidation.

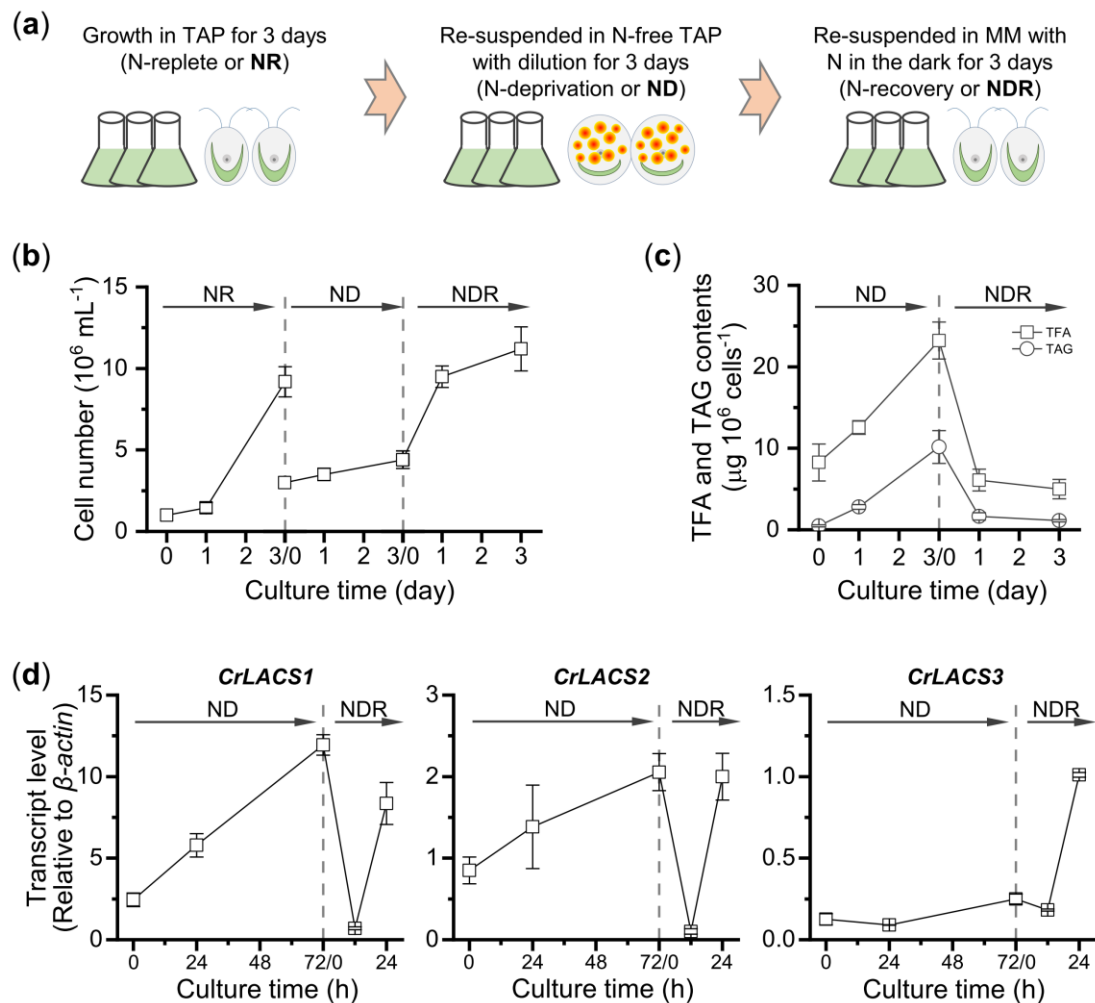
257

258 **Dynamic growth, TAG levels and transcriptional expression of *CrLACS* genes in**
 259 ***Chlamydomonas* in response to changing N status**

260 N-deprivation is one of the most well studied stimuli for inducing TAG synthesis in
 261 algae (Valledor *et al.*, 2014; Park *et al.*, 2015; Liu *et al.*, 2016a; Liu *et al.*, 2019). To
 262 understand the role of CrLACS members in cell growth and TAG metabolism,
 263 *Chlamydomonas* cells were first cultured in TAP medium (N-replete or NR condition),
 264 then in N-free TAP medium (N-deprivation or ND condition) to induce TAG synthesis
 265 and finally in the minimal medium (with N but without acetate) in the dark (N-recovery
 266 or NDR condition) for TAG remobilization (Fig. 1a). *Chlamydomonas* grew fast during
 267 NR, followed by severely impaired growth during ND and rapid regrowth during NDR
 268 (Fig. 1b). Total fatty acid (TFA) content rose upon ND and reached a 2.8-fold increase
 269 after 3 days, suggesting active *de novo* fatty acid synthesis. A decline in TFAs during
 270 NDR was observed, indicating the occurrence of fatty acid catabolism (Fig. 1c).
 271 Similarly, TAG content increased upon ND yet to a much greater extent (21.2-fold) and
 272 then declined under NDR (Fig. 1c). The NDR-associated TAG decline is attributed to
 273 TAG catabolism.

274 Upon ND, *CrLACS1* exhibited a considerable up-regulation and its transcript level
 275 at 72 h reached 4.9-fold greater than that at 0 h; in response to NDR treatment, *CrLACS1*
 276 was dramatically down-regulated within 12 h (Fig. 1d). Similar to *CrLACS1*, *CrLACS2*
 277 was up-regulated by ND and showed a rapid down-regulation within 12 h of NDR (Fig.
 278 1d). *CrLACS3*, differing from *CrLACS1* and *CrLACS2*, had slight transcriptional

279 changes during the ND period; upon NDR after 12 h, *CrLACS3* was up-regulated
 280 substantially (Fig. 1d). Taken together, the dynamics of TAG content and *CrLACS*s'
 281 transcriptional expression pattern suggest that *CrLACS1* and *CrLACS2* are likely
 282 involved in TAG biosynthesis while *CrLACS3* in TAG catabolism. Interestingly,
 283 *CrLACS1* and *CrLACS2* also exhibited a transcriptional up-regulation after 12 h of
 284 NDR (Fig. 1d). Probably, cell regrowth begins after 12 h of NDR and needs
 285 regeneration of enough membrane lipids, in which *CrLACS1* and *CrLACS2* are required.



286

287 **Fig. 1** Growth, lipid levels and transcriptional expression of *CrLACS* genes in
 288 *Chlamydomonas* under various N status. (a) Schematic illustration of three-stage
 289 culture conditions (sequentially N replete - NR, N deprivation - ND, then N deprivation-
 290 then-recovery - NDR). (b) Cell numbers. (c) TFA and TAG contents. (d) Transcript
 291 levels of *CrLACS1*, *CrLACS2* and *CrLACS3* relative to the internal control gene β -actin,
 292 quantified by RT-qPCR. MM, minimal medium without acetate. Data represent mean \pm

293 SD ($n=3$).

294

295 **Heterologous expression of *CrLACS1*, *CrLACS2* and *CrLACS3* each recovered the**
296 **ability of the yeast *faa1 faa4* double mutant to grow on FFAs**

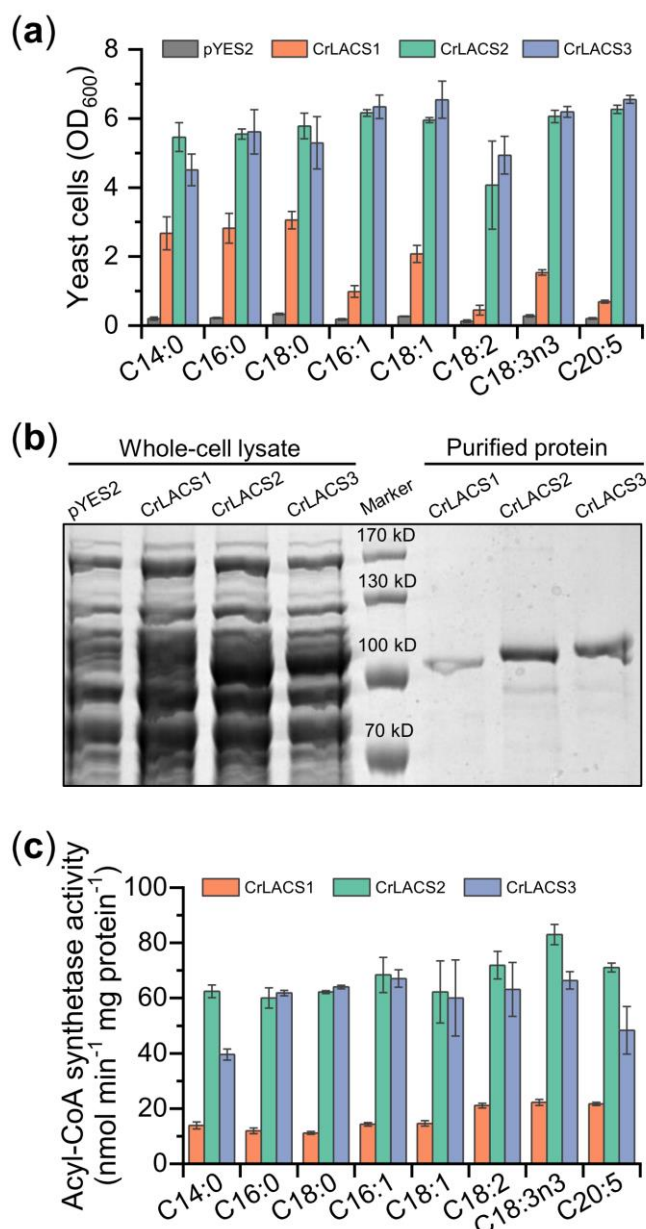
297 To validate the function of *CrLACS* genes, their coding sequences were each introduced
298 into the *Saccharomyces cerevisiae* strain YB525 for complementation. YB525,
299 disrupted in the two LACS-coding genes *FAA1* and *FAA4*, is unable to grow with
300 exogenous free fatty acids (FFAs) as the sole carbon source and thus has been used for
301 functional studies of putative *LACS* genes (Knoll *et al.*, 1995). Eight FFAs were
302 examined, with the carbon chain length ranging from 14 to 20 and the double bonds
303 from 0 to 5. Unlike the control (pYES2) that barely grew, *CrLACS1*, *CrLACS2* and
304 *CrLACS3* all enabled the yeast cells to grow on any of the eight tested FFAs (Fig. 2a),
305 suggesting that their encoded proteins all showed LACS activity when expressed in the
306 yeast. However, the yeast strains expressing *CrLACS2* and *CrLACS3* achieved
307 comparable cell densities and were considerably greater than that expressing *CrLACS1*,
308 indicating *CrLACS2* and *CrLACS3* may possess stronger enzymatic activities than
309 *CrLACS1*.

310

311 **The recombinant *CrLACS1*, *CrLACS2* and *CrLACS3* all exhibited acyl-CoA**
312 **synthetase activities**

313 To gain direct evidence with respect to the activity and substrate preference of
314 *CrLACS*s, each recombinant *CrLACS* protein expressed in *E. coli* was purified for *in*
315 *vitro* enzymatic activity assays, using the above-mentioned FFAs as substrates (Fig.
316 2b,c). Consistent with the FFA feeding experiments (Fig. 2a), *CrLACS2* and *CrLACS3*
317 had comparable enzymatic activities and were superior to *CrLACS1* regardless of the
318 FFA substrates (Fig. 2c). It is worth noting here that each of *CrLACS1*, *CrLACS2* and
319 *CrLACS3* had activities on all eight tested FFAs and exhibited little substrate
320 preference (Fig. 2c). This forms clear contrast to the *C. zofingiensis* LACS isozymes
321 which showed distinct substrate (i.e. FFAs) preferences both *in vitro* and in the yeast

322 complementation experiment (Wu *et al.*, 2020).



323

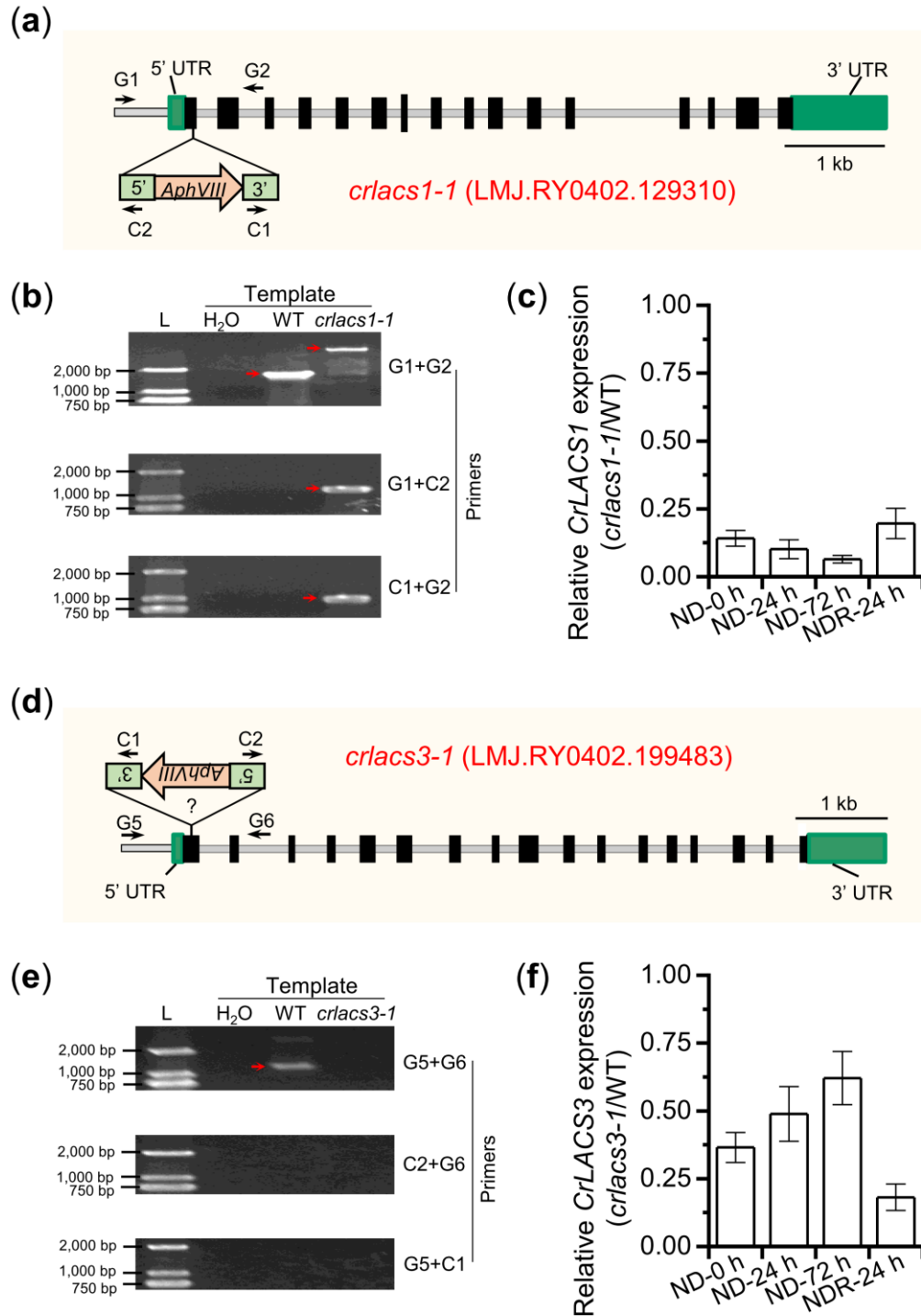
324 **Fig. 2** Functional characterization of CrLACS1, CrLACS2 and CrLACS3. (a) Growth
 325 of YB525 cells carrying the empty vector pYES2 or *CrLACS* genes fed on various free
 326 fatty acids (FFAs). Yeast growth was recorded at OD 600 nm after 10 days of cultivation.
 327 (b) SDS-PAGE separation of recombinant CrLACS1, CrLACS2 and CrLACS3 proteins
 328 from *E. coli*. (c) *In vitro* enzymatic assay of CrLACS proteins on various FFAs. Data
 329 in (a, c) represent mean \pm SD ($n=3$).

330

331 **Molecular characterization of insertional mutants of CrLACS1 and CrLACS3**

332 We further investigated the functions of the three LACSs in *Chlamydomonas* via
333 analysis of insertional mutants. Several insertional lines for each of *CrLACS1*,
334 *CrLACS2* and *CrLACS3* could be identified in the *Chlamydomonas* mutant library
335 (CLIP collection) (Li *et al.*, 2016). Two putative mutants for *CrLACS1*,
336 LMJ.RY0402.129310 (*crlacs1-1*) and LMJ.RY0402.173140 (*crlacs1-2*) were selected
337 for molecular characterization. Using genomic DNA as the template, PCR amplification
338 and Sanger sequencing confirmed insertion of the cassette (with intact 5' and 3' ends)
339 in sense orientation in the first exon of *CrLACS1* for *crlacs1-1* (Fig. 3a, b) and in
340 antisense orientation in the ninth exon for *crlacs1-2* (Fig. S3a, b). Compared to WT,
341 *crlacs1-1* and *crlacs1-2* had ~15.4-fold and 9.0-fold lower *CrLACS1* transcript,
342 respectively (Fig. 3c and Fig. S3c).

343 Since the *CrLACS2* mutant LMJ.SG0182.015843 had been characterized
344 previously (Li *et al.*, 2016), it, after further molecular confirmation (Fig. S4), was
345 designated as *crlacs2-1* here and used for growth and lipid analyses in our study. As for
346 *CrLACS3*, LMJ.RY0402.199483 (*crlacs3-1*) was chosen for molecular characterization.
347 Although genomic PCR amplification suggested insertion in the expected sites of
348 *CrLACS3*, the 5' and 3' ends of the cassette were not recovered (Fig. 3d, e). This may
349 be due to deletions in the junctions between the cassette and insertion site (Li *et al.*,
350 2016). Nevertheless, ~5.5-fold decrease of *CrLACS3* transcript was observed in
351 *crlacs3-1* as compared with WT (Fig. 3f).



352

353 **Fig. 3** Molecular characterization of the insertional mutants *crlacs1-1* and *crlacs3-1*. (a,
 354 b) Schematic map (a) and PCR validation (b) of the insertion site in *CrLACS1* for
 355 *crlacs1-1*. (c) Transcriptional levels of *CrLACS1* in *crlacs1-1* relative to WT. (d, e)
 356 Schematic map (d) and PCR validation (e) of the insertion site in *CrLACS3* for *crlacs3-*
 357 *1*. (f) Transcriptional levels of *CrLACS3* in *crlacs3-1* relative to WT. Arrows in (a, d)

358 designate primers used for validating insertion of the cassette. Data in (c, f) represent
 359 mean \pm SD ($n=3$).

360

361 ***CrLACS1* or *CrLACS2* suppression attenuated TAG synthesis under N-deprivation**

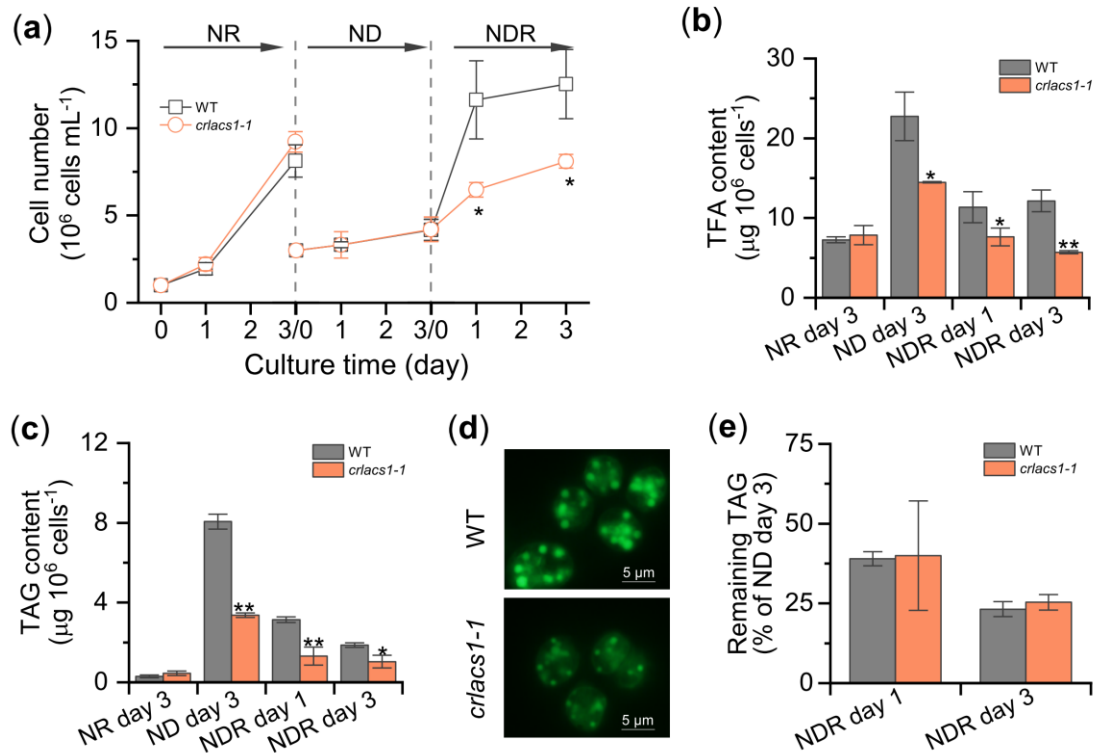
362 There was no obvious difference in growth between *crlacs1-1* and WT under NR or
 363 ND condition, as indicated by the almost identical cell density (Fig. 4a) and dry weight
 364 per 10^6 cells or per mL (Fig. S5a). In response to NDR, cell division was followed and
 365 we observed that *crlacs1-1* reached a lower cell density than WT, suggesting an
 366 impaired cell division in the mutant (Fig. 4a). Accordingly, *crlacs1-1* had a greater dry
 367 weight per cell than WT under this condition (Fig. S5a). TFA and TAG contents rose
 368 upon ND and recovered upon NDR in both *crlacs1-1* and WT (Fig. 4b, c). It is worth
 369 noting here that *CrLACS1* suppression caused a reduction in both TFA and TAG
 370 contents under ND (day 3), but not under NR (day 3); the difference was also noticed
 371 under NDR condition (Fig. 4b, c). As such, *crlacs1-1* had a weaker green fluorescence
 372 intensity than WT when stained with the neutral lipids specific dye Bodipy (Fig. 4d).

373 Interestingly, albeit *crlacs1-1* had a lower TAG content than WT under NDR
 374 condition, they showed little difference in the TAG remobilization rate as revealed by
 375 the comparable percentage of remaining TAG (normalized to TAG content on day 3 of
 376 ND) (Fig. 4e). Nevertheless, the total amount of TAG being remobilized was less in the
 377 mutant compared to WT, explaining the reduced capacity in cell regrowth in the mutant.

378 In addition, regardless of the culture conditions, only slight variations were
 379 observed between *crlacs1-1* and WT in the fatty acid composition of either TFA or TAG
 380 (Fig. S6), consistent with the unbiased preference of *CrLACS1* for FFAs (Fig. 1c). The
 381 second mutant allele *crlacs1-2* showed similar phenotypes e.g. lower TFA and TAG
 382 levels than WT (Fig. S3d). Taken together, our data demonstrated that *CrLACS1* is
 383 involved in TAG biosynthesis rather than TAG catabolism in *Chlamydomonas*.

384 Similarly, the growth of *crlacs2-1* was comparable to WT under NR and ND
 385 conditions but was lower than WT under NDR condition (Fig. S7a). Moreover, *crlacs2-1*
 386 exhibited lower TFA and TAG contents than WT under ND (day 3) and NDR

387 conditions (Fig. S7b-d), yet little difference was observed between them in the fatty
 388 acid composition of either TFA or TAG (Fig. S8) and in the TAG remobilization ability
 389 (Fig. S7e). In this context, *CrLACS2*, resembling *CrLACS1*, contributes to TAG
 390 biosynthesis instead of TAG catabolism.



391

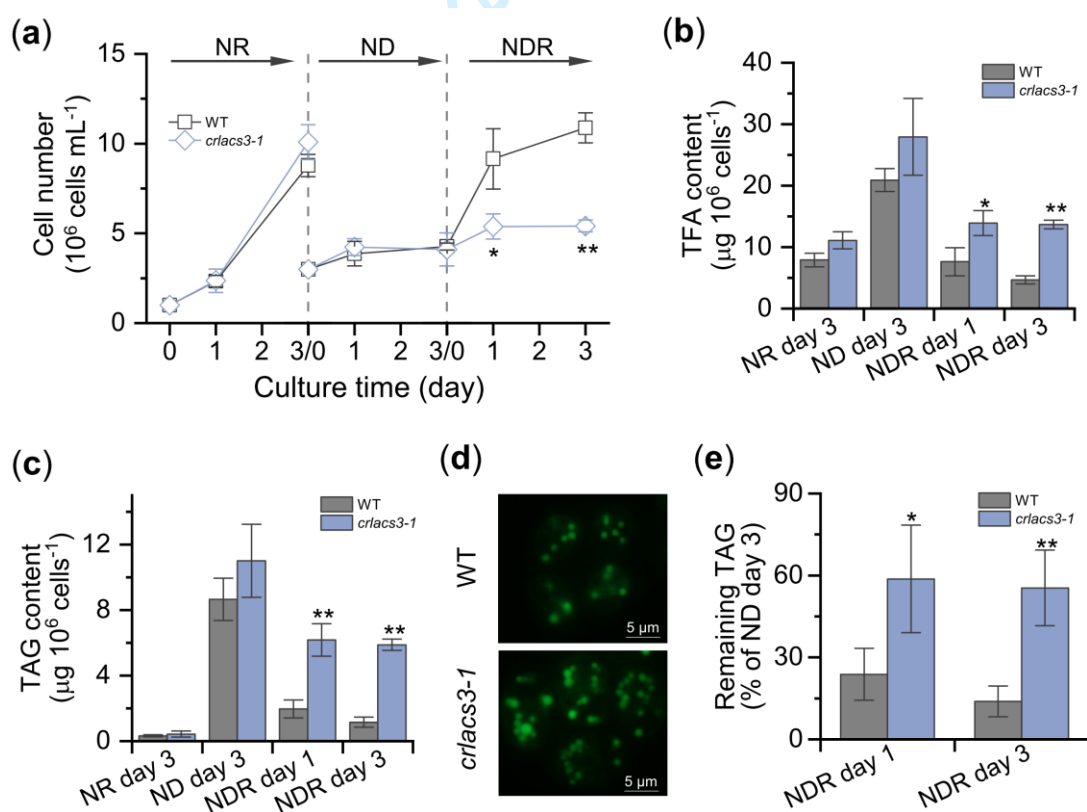
392 **Fig. 4** Comparison of growth and lipids between WT and *crlacs1-1* cells under NR, ND
 393 and NDR conditions. (a) Cell number per mL. (b) TFA content. (c) TAG content. (d)
 394 Fluorescent microscopy observation of Bodipy-stained algal cells (ND day 3). Green
 395 signals indicate lipid droplets. (e) Percentage of remaining TAG under NDR conditions
 396 (relative to ND day 3). Data in (a-c, f) represent mean \pm SD ($n=3$). Asterisks indicate
 397 the significant difference (Student's *t*-test, $P<0.05^*$ or $P<0.01^{**}$).

398

399 ***CrLACS3* suppression impaired TAG remobilization and cell growth severely** 400 **during N recovery**

401 Under NR and ND conditions, *crlacs3-1* and WT had comparable cell density and
 402 showed no growth difference; upon NDR, while WT underwent a rapid cell division,
 403 *crlacs3-1* was severely impaired and its cell density was kept at a low but relatively
 404 stable level during the 3-day NDR period (Fig. 5a). Albeit not significant, *crlacs3-1* had

405 a slightly higher TFA content than WT during ND period; upon NDR, the difference in
 406 TFA content between *crLACS3-1* and WT increased (Figure 5b). TAG content in *crLACS3-*
 407 *1* was also slightly greater than that in WT under ND; upon NDR, while WT exhibited
 408 a drastic TAG decrease, *crLACS3-1* was severely impaired in TAG decline i.e. its TAG
 409 content was 5.1-fold higher than that in WT on day 3 of NDR (Fig. 5c). The large
 410 difference in TAG content under NDR between *crLACS3-1* and WT was also supported
 411 by the fluorescence microscopic observation of Bodipy-stained cells (Fig. 5d).
 412 *CrLACS3* suppression therefore severely disturbed the TAG remobilization ability, as
 413 revealed by the considerably greater percentage of remaining TAG in *crLACS3-1* under
 414 NDR (Fig. 5e). Similar to *CrLACS1* or *CrLACS2* (Fig. S6 and S8), *CrLACS3*
 415 suppression led to little difference in the fatty acid composition of either TFA or TAG
 416 (Fig. S9). Taken together, *CrLACS3*, in contrast to *CrLACS1* or *CrLACS2*, is involved
 417 in TAG catabolism instead of TAG biosynthesis in *Chlamydomonas*.



418

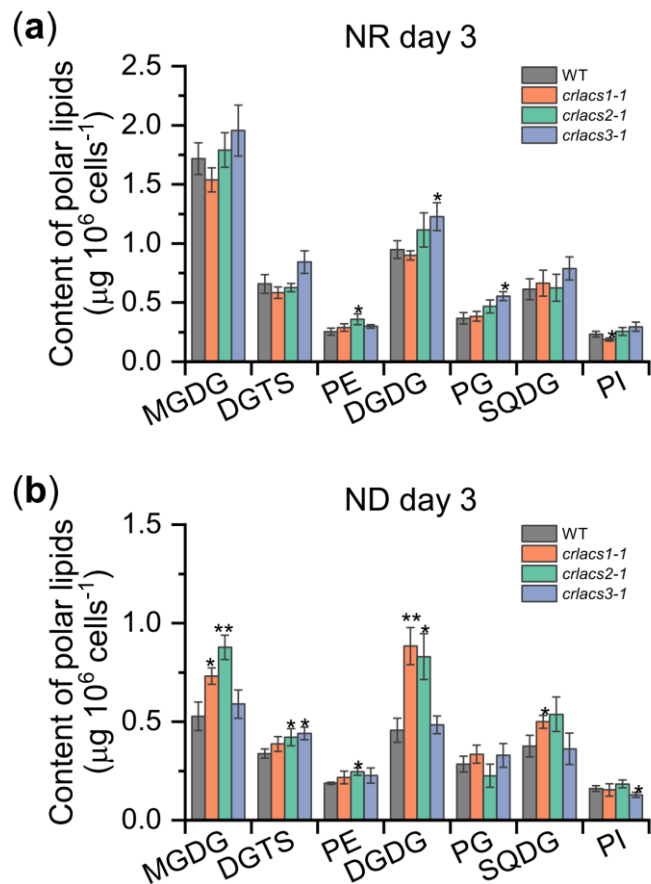
419 **Fig. 5** Comparison of growth and lipids between WT and *crLACS3-1* cells under NR, ND
 420 and NDR conditions. (a) Cell number per mL. (b) TFA content. (c) TAG content. (d)
 421 Fluorescent microscopy observation of Bodipy-stained algal cells (day 3 of NDR).

422 Green signals indicate lipid droplets. (e) Percentage of remaining TAG under NDR
423 conditions (relative to ND day 3). Data in (a-c, f) represent mean \pm SD ($n=3$). Asterisks
424 indicate the significant difference (Student's *t*-test, $P<0.05^*$ or $P<0.01^{**}$).

425

426 **Suppression of CrLACS1 or CrLACS2 but not CrLACS3 led to more galactolipids**
427 **during N-deprivation**

428 TAG amount in N-deprived *Chlamydomonas* cells is partly due to *de novo* synthesis
429 and partly due to membrane lipid remodeling. To determine how the suppression of
430 *CrLACS* genes affected polar membrane lipids, monogalactosyldiacylglycerol
431 (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyl diacylglycerol
432 (SQDG), phosphatidylglycerol (PG), phosphatidyl inositol (PI),
433 phosphatidylethanolamine (PE) and diacylglycerol trimethylhomoserine (DGTS) were
434 quantified for WT, *crlacs1-1*, *crlacs2-1* and *crlacs3-1*. Under NR condition, only
435 slight/little differences in the contents of polar lipids were observed between WT and
436 the mutants (Fig. 6a), suggesting that disruption of the individual *CrLACS* genes had
437 limited effect on metabolism of polar lipids under favorable growth conditions.
438 Nevertheless, on day 3 of ND when TAG was induced to accumulate, different
439 responses were observed in the three mutants: compared to WT, both *crlacs1-1* and
440 *crlacs2-1* contained higher levels of chloroplast membrane lipids particularly MGDG
441 and DGDG but not the ER membrane lipids, while *crlacs3-1* exhibited little difference
442 in any of the membrane polar lipids (Fig. 6b). This result suggests that CrLACS1 and
443 CrLACS2 participate in the activation of FFAs, coming from galactolipid hydrolysis,
444 for TAG synthesis; in their absence, TAG amount is reduced, and galactolipid turnover
445 is halted likely as a result of feedback inhibition by the FFAs.



446

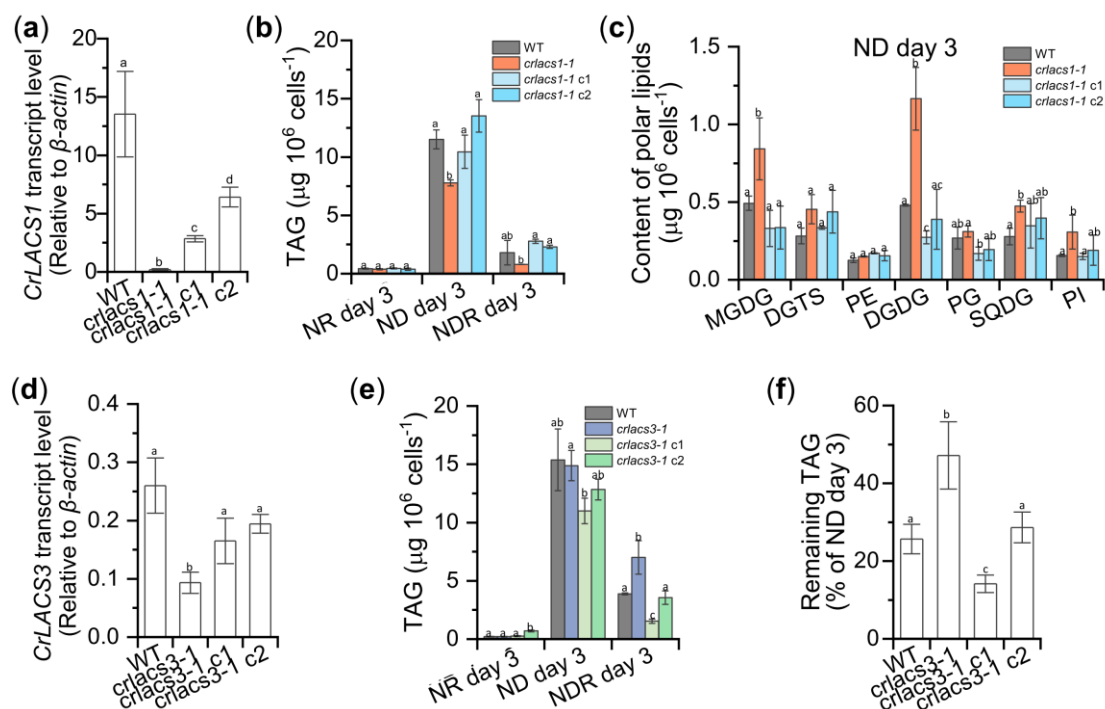
447 **Fig. 6** Contents of membrane lipid classes in WT and mutants under NR and ND
 448 conditions. (a) NR day 3. (b) ND day 3. Data represent mean \pm SD ($n=3$). Asterisks
 449 indicate the significant difference (Student's t -test, $P<0.05^*$ or $P<0.01^{**}$).

450

451 Genetic complementation of *CrLACS1* and *CrLACS3* insertional mutants

452 To confirm whether the phenotypes of *crlacs1-1* and *crlacs3-1* were caused by
 453 suppression of *CrLACS1* and *CrLACS3*, respectively, complementation experiments
 454 were performed. Functional complementation of *crlacs2-1* was previously performed
 455 (Li *et al.*, 2016) and thus not repeated here. Two independent complemented strains for
 456 each mutant were analyzed. Clearly, introduction of *CrLACS1* into *crlacs1-1* restored
 457 the transcriptional expression of *CrLACS1* (Fig. 7a), TAG content under ND (day 3)
 458 and NDR (day 3) conditions (Fig. 7b), and MGDG and DGDG contents (Fig. 7c),
 459 demonstrating firmly that the phenotypes of *crlacs1-1* were due to *CrLACS1*
 460 dysfunction. Introduction of *CrLACS3* into *crlacs3-1* also led to restoration of
 461 *CrLACS1* transcriptional expression (Fig. 7d), TAG content under NDR (day 3)

462 condition (Fig. 7e) and TAG remobilization ability (Fig. 7f), further confirming the
 463 function of CrLACS3 in TAG catabolism in *Chlamydomonas*.



464

465 **Fig. 7** Complementation of *crlacs1-1* and *crlacs3-1* strains. (a) The transcript levels of
 466 *CrLACS1* (relative to β -actin) in WT, *crlacs1-1* and the complemented strains on day 3
 467 of ND as determined by RT-qPCR. (b) TAG contents in WT, *crlacs1-1* and the
 468 complemented strains under NR, ND and NDR conditions. (c) Contents of polar lipids
 469 in WT, *crlacs1-1* and the complemented strains on day 3 of ND. (d) The transcript levels
 470 of *CrLACS3* (relative to β -actin) in WT, *crlacs3-1* and the complemented strains on day
 471 3 of NDR as determined by RT-qPCR. (e) TAG contents in WT, *crlacs3-1* and the
 472 complemented strains under ND and NDR conditions. (f) Percentage of remaining TAG
 473 on day 3 of NDR (relative to ND day 3) in WT, *crlacs3-1* and the complemented strains.
 474 Data represent mean \pm SD ($n=3$). Different letters above the bars in each group indicate
 475 significant difference ($p<0.05$), based on one-way analysis of variance (ANOVA) and
 476 Tukey's honestly significant difference (HSD) test.

477

478 **Comparative transcriptomic analysis between mutants and WT**

479 To understand the transcriptional responses caused by insertional suppression of

480 *CrLACS* genes, comparative RNA sequencing (RNA-seq) analysis was performed for
481 WT, *crlacs1-1* and *crlacs2-1* under ND condition and for WT and *crlacs3-1* under NDR
482 condition. Ten high-quality transcriptomes were generated and the biological duplicates
483 within each group showed a high consistence and distinguished from other groups as
484 indicated by the Pearson correlation and principal component analysis (Fig. S10a, b).
485 Over 17,000 genes were mapped and the majority of them had a FPKM value no less
486 than 1 (Fig. S10c and Data S1). Although significant changes occurred for TAG in
487 *crlacs1-1* and *crlacs2-1* under ND conditions (Fig. 4 and Fig. S7) and in *crlacs3-1*
488 under NDR conditions (Fig. 5), the majority of genes involved in lipid metabolic
489 pathways such as *de novo* fatty acid synthesis, fatty acid desaturation, membrane lipid
490 synthesis and turnover (e.g., the MGDG lipase PLASTID
491 GALACTOGLYCEROLIPID DEGRADATION1 - PGD1), TAG assembly and
492 degradation (e.g., the TAG lipase LIP4), and fatty acid β -oxidation (e.g., the acyl-CoA
493 oxidase 2 - ACX2) showed only slight transcriptional variations as compared with WT
494 (Data S2). Likely, the phenotypes of *crlacs1-1*, *crlacs2-1* and *crlacs3-1* observed at the
495 level of lipid classes are therefore mainly attributed to metabolic adjustment rather than
496 transcriptional reprogramming.

497

498 Discussion

499 **CrLACS1, CrLACS2 and CrLACS3 are bona fide long-chain acyl-CoA** 500 **synthetases with activity on a broad range of FFAs**

501 In this study, we demonstrated clearly that CrLACS1, CrLACS2 and CrLACS3 are
502 bona fide LACS enzymes and have overlapping activities in activating FFAs (Fig. 1c).
503 Albeit CrLACS1 has considerably lower activities than CrLACS2 or CrLACS3, they
504 all are functional on a broad range of FFAs without obvious substrate preference. By
505 contrast, in *C. zofingiensis*, an oleaginous green alga closely related to Chlamydomonas
506 (Zhang *et al.*, 2021), the LACS enzymes exhibit distinct preference on FFAs with
507 different carbon chain length and double bonds (Wu *et al.*, 2020). Specifically,
508 CzLACS2, a close homolog of CrLACS2 with 59.5% sequence identity, acts on

509 saturated and monounsaturated fatty acids yet has little activities on polyunsaturated
510 fatty acids. This suggests that even being phylogenetically closely related, LACS
511 homologues from different algal species may vary greatly in their substrate preference.
512 On the other hand, CzLACS5, a close homolog of CrLACS3 with 69.6% sequence
513 identity, resembles CrLACS3 and acts on a broad range of FFAs. As both
514 *Chlamydomonas* and *C. zofingiensis* harbor only one peroxisomal LACS isoform (Li-
515 Beisson *et al.*, 2015; Wu *et al.*, 2020), it is not surprising that they did not show
516 particular substrate preference, which is important in ensuring the catabolism of all
517 FFAs by the fatty acid β -oxidation in the peroxisomes.

518

519 **CrLACS1 and CrLACS2 functionally overlap in TAG biosynthesis and**
520 **participate in allocation of acyl chains between TAG and galactolipids**

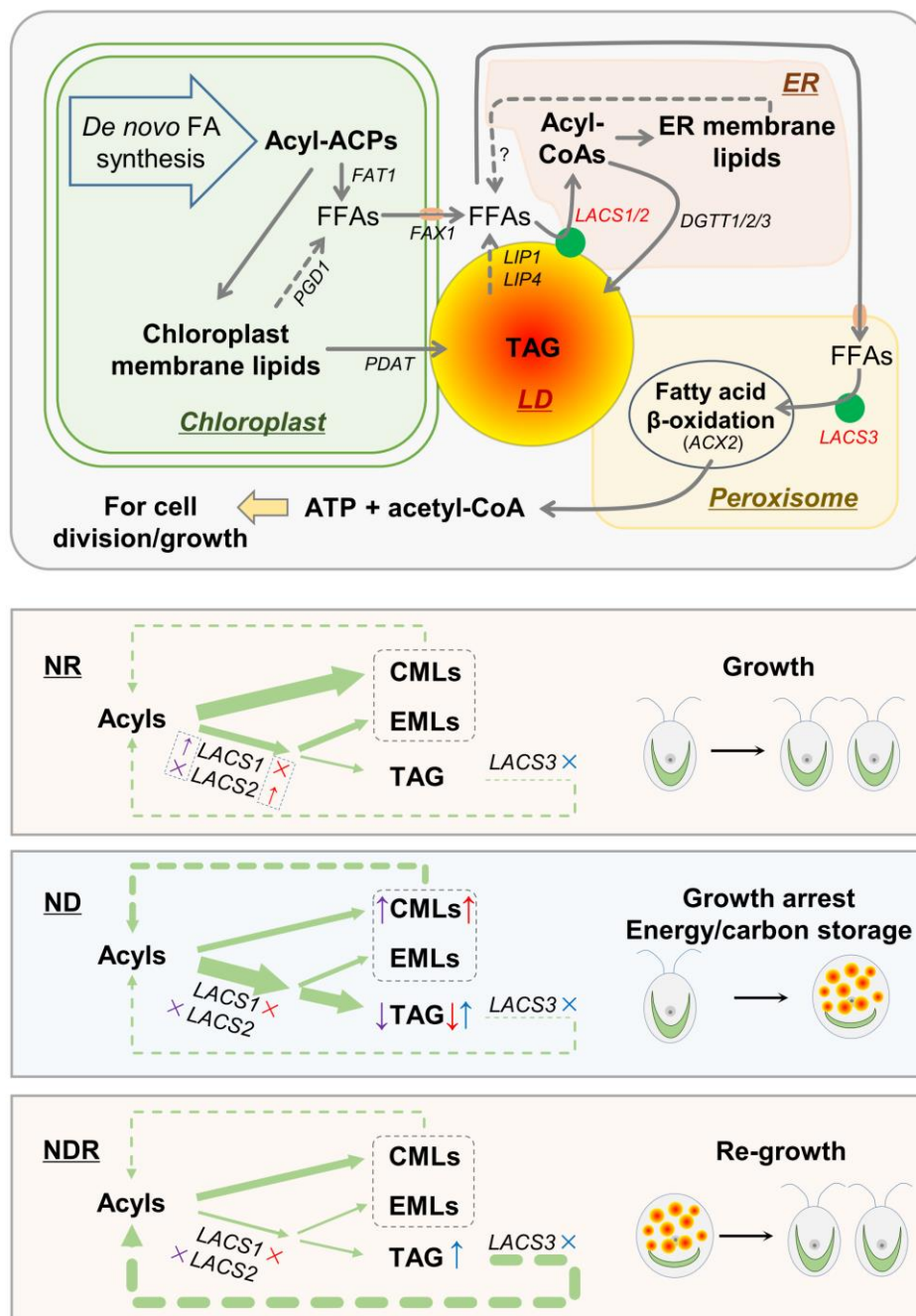
521 LACS is known to participate in a variety of anabolic and catabolic pathways related
522 to acyl-lipid metabolism (DiRusso & Black, 1999; Coleman *et al.*, 2002; Watkins &
523 Ellis, 2012; Zhao *et al.*, 2019). In *Chlamydomonas*, upon N-deprivation, a stimulus
524 widely used for inducing TAG accumulation, our RT-qPCR results showed that both
525 *CrLACS1* and *CrLACS2* were considerably up-regulated (Fig. 1d), consistent with
526 large-scale RNA-seq studies (Miller *et al.*, 2010; Boyle *et al.*, 2012; Blaby *et al.*, 2013;
527 Goodenough *et al.*, 2014; López García de Lomana *et al.*, 2015). Moreover, when N-
528 deprivation stress was removed (NDR condition), TAG level declined, accompanied by
529 the transcriptional down-regulation of *CrLACS1* and *CrLACS2* (Fig. 1c, d). These
530 correlations point to the potential involvement of *CrLACS1* and *CrLACS2* in TAG
531 biosynthesis. As a strong support, it has been reported that insertional mutation of
532 *CrLACS2* impaired TAG level in *Chlamydomonas* under N-deprivation condition (Li
533 *et al.*, 2016). Nevertheless, it did not block TAG accumulation; instead, about half of
534 TAG was maintained, suggesting that additional LACS gene(s) are involved in TAG
535 biosynthesis in *Chlamydomonas*. In the present study, we quantified TAG levels in
536 *CrLACS1* mutants as well as the complemented strains (Fig. 4c and 7b), and the results
537 firmly demonstrated that CrLACS1 is also involved in TAG biosynthesis. Although

538 CrLACS1 has weaker *in vitro* enzymatic activities than CrLACS2 (Fig. 1c), its greater
539 transcriptional increase upon N-deprivation (Fig. 2d) may have boosted their activities
540 substantially. Considering that suppression of CrLACS1 or CrLACS2 led to ~50% TAG
541 reduction, they two likely make comparable contribution to TAG synthesis in
542 Chlamydomonas and function independently of each other. In accord with this, at least
543 at the transcriptional level, neither *CrLACS1* nor *CrLACS2* disruption stimulated the
544 expression of the other one during N-deprivation (Fig. S11).

545 We attempted to generate double mutants of *CrLACS1* and *CrLACS2* by genetic
546 crossing, but failed, likely because the double mutants are lethal. It is reasonable as
547 acyl-CoAs produced from the action of CrLACS1 and CrLACS2 also participate in the
548 synthesis of extra-chloroplast membrane lipids (Li-Beisson *et al.*, 2015), which are
549 essential for cell survival. While suppression of *CrLACS1* or *CrLACS2* attenuated TAG
550 levels on day 3 of N-deprivation (Fig. 4c and Fig. S6c), the ER membrane lipids DGTS,
551 PE and PI showed little variations (Fig. 6b). This is not surprising considering that
552 during N-deprivation, membrane lipid syntheses were halted thus there is little
553 requirement for acyl-CoAs. It is worth noting here that in comparison with WT, both
554 *crlacs1-1* and *crlacs2-1* maintained higher levels of the chloroplast lipids MGDG and
555 DGDG under N-deprivation (Fig. 6b), pointing to a role of CrLACS1 and CrLACS2 in
556 allocating acyl chains between TAG and chloroplast lipids particularly galactolipids.
557 These results also support that galactolipid remodeling contributes fatty acids for TAG
558 biosynthesis in Chlamydomonas, in line with the previous study where the insertional
559 mutation of *PGDI* gene that encodes a MGDG lipase impairs TAG accumulation (Li *et*
560 *al.*, 2012b). The fatty acids either *de novo* synthesized or recycled from chloroplast
561 lipids, prior to incorporation into TAG, have to be exported out of the chloroplast and
562 activated by LACS enzymes. It has been reported that LACS, coupled with a fatty acid
563 transporter, is involved in transporting FFAs into the cytoplasm of bacteria and yeast
564 cells (DiRusso & Black, 1999; Zou *et al.*, 2003). Similarly, in land plants and algae,
565 LACS probably functions with a fatty acid transporter (FAX1) localized at the
566 chloroplast envelop (Li *et al.*, 2015; Li *et al.*, 2019) and facilitate transport of FFAs

567 from the chloroplast to the cytoplasm. In this context, suppression of *CrLACS1* or
568 *CrLACS2* may impair export of FFAs out the chloroplast, which attenuates galactolipid
569 turnover (e.g., mediated by *PGD1*) and hydrolysis of acyl-ACPs (i.e., catalyzed by the
570 acyl-ACP thioesterase *FAT1*) via the possible product feedback inhibition by the FFAs
571 (Fig. 8). Accordingly, more acyl-ACPs may be rerouted to chloroplast membrane lipids.
572 Nevertheless, the transcriptional levels of *PGD1*, *FAT1*, or *FAX1* were not down-
573 regulated in *crlacs1-1* or *crlacs2-1* as compared to WT (Data S2), indicating that the
574 lipid phenotypes are likely due to a shift in metabolic adjustment rather than
575 transcriptional reprogramming. It has been reported in *Arabidopsis* that impairing
576 export of FFAs out the chloroplast also causes increased levels of certain chloroplast
577 membrane lipids at the expense of TAG (Li *et al.*, 2015).

578 Nevertheless, when *Chlamydomonas* grew during N-replete condition and it
579 synthesized a basal level of TAG, neither TAG nor any of the chloroplast membrane
580 lipids was impacted by *CrLACS1* or *CrLACS2* suppression (Fig. 4c, 6a and S7c). We
581 examined expression of *CrLACS* genes under N-replete condition in *crlacs1-1* and
582 *crlacs2-1*; interestingly, *CrLACS1* suppression caused a 5.1-fold increase of *CrLACS2*
583 transcript while *CrLACS2* suppression led to a 2.2-fold increase of *CrLACS1* transcript
584 (Fig. S11). In other words, when the individual *CrLACS1* or *CrLACS2* is inactivated,
585 *Chlamydomonas* can adopt a compensation strategy to maintain lipid homeostasis by
586 stimulating expression of the other. It is worth mentioning that this compensation
587 strategy occurs under N-replete but not N-deprivation (Fig. S11), indicating a fine
588 temporal collaboration between *CrLACS1* and *CrLACS2*. Of course, we cannot exclude
589 the possibility that the acyl-CoA independent pathway mediated by the
590 phospholipid:diacylglycerol acyltransferase (*PDAT*) could be further stimulated to
591 make TAG in response to *CrLACS1* or *CrLACS2* disruption under N-replete condition,
592 as the *Chlamydomonas* *PDAT* is known to preferentially function under favorable
593 growth conditions (Yoon *et al.*, 2012).



594

595 **Fig. 8** Proposed model explaining that CrLACS1, CrLACS2 and CrLACS3 function
 596 spatially and temporally to achieve lipid homeostasis for growth and energy storage in
 597 Chlamydomonas. Multiple organelles are involved in lipid metabolism, including the
 598 chloroplast, ER, LDs and peroxisomes, etc. CrLACS1 and CrLACS2 reside in the ER
 599 and/or LDs, and contribute to TAG biosynthesis, while CrLACS3 is localized in
 600 peroxisomes and contributes to TAG degradation. Under NR condition, growth is active
 601 for cell reproduction; acyls are allocated predominantly to chloroplast membrane lipids

602 (CMLs) and ER membrane lipids (EMLs), with a basal level of TAG. Under ND
603 condition, growth is arrested; membrane lipid remodeling and TAG biosynthesis are
604 stimulated while TAG catabolism is minimized, leading to massive accumulation of
605 TAG for energy and carbon storage. Suppression of *CrLACS1* and *CrLACS2* each
606 impairs TAG accumulation with increased levels of CMLs particularly MGDG and
607 DGDG. Exposing ND-treated cells to NDR condition, TAG biosynthesis is minimized
608 while TAG catabolism is stimulated, resulting in TAG degradation to supply carbon and
609 energy sources for cell re-growth. *CrLACS3* suppression severely impairs TAG
610 degradation and thus the cell re-growth. The thickness of green arrows refer to acyl
611 fluxes (not on scale).

612

613 **CrLACS3 plays a critical role in TAG remobilization to support cell growth**

614 In *Chlamydomonas*, fatty acid β -oxidation occurs in peroxisomes, which play a critical
615 role in remobilizing the energy-dense TAG and providing energy and carbon precursors
616 for cell division (Kong *et al.*, 2017; Kong *et al.*, 2018; Lee *et al.*, 2020). *CrLACS3*
617 suppression severely impaired TAG degradation and allowed the alga to maintain high
618 levels of TAG during N-recovery (Fig. 5c). CrLACS3, predicted to reside in
619 peroxisomes based on homology to other known LACS proteins, thus connects TAG
620 degradation to fatty acid β -oxidation by activating FFAs released from TAG and DAG
621 that are catalyzed by LIP4 (Warakanont *et al.*, 2019) and LIP1 (Li *et al.*, 2012a),
622 respectively (Fig. 8). Moreover the defects in TAG catabolism is accompanied by a near
623 total block in cell growth (Fig. 5a), suggesting a critical role of *CrLACS3* in TAG
624 remobilization for supporting cell division when the adverse conditions are relieved. It
625 is worth mentioning here that in *crlacs3-1* TAG remobilization was not completely
626 blocked, which is most likely due to the fact that *crlacs3-1* is only a knock-down rather
627 than a knock-out mutant. The function of CrLACS3 is further supported by its dynamic
628 expression pattern at the transcriptional level during varying N status (Fig. 2d).
629 Moreover, the expression profile of *CrLACS3* also agrees with the transcriptional
630 expression patterns of known lipolytic enzymes such as LIP4 and LIP1 or the known

631 peroxisomal ACX2 that catalyzes the first step of fatty acid β -oxidation in
632 *Chlamydomonas* (Li *et al.*, 2012b; Kong *et al.*, 2017; Warakanont *et al.*, 2019). These
633 points to an orchestrated transcriptional control of TAG remobilization.

634 The remobilization of TAG to provide carbon and energy sources for cell regrowth
635 is reflected by suppression of not only *CrLACS3* but also *CrLACS1* or *CrLACS2*. Under
636 N-deprivation (ND day 3), both *crlacs1-1* and *crlacs2-1* have lower amounts of TAG
637 than the WT (Fig.4c and Fig. S7c). When subjected to N-recovery, although the TAG
638 remobilization rate is not affected, the amounts of remobilized TAG in *crlacs1-1* and
639 *crlacs2-1* are smaller than that in WT (Fig.4e and Fig. S7e). Therefore, less carbon and
640 energy sources are available for the mutants, leading to impaired cell growth (Fig.4a
641 and Fig. S7a).

642

643 **CrLACS1, CrLACS2 and CrLACS3 collaborate spatially and temporally in lipid** 644 **homeostasis**

645 Multiple organelles are involved in lipid metabolism, including chloroplast, ER, LDs
646 and peroxisomes. Serving as connections among these organelles, CrLACS1,
647 CrLACS2 and CrLACS3 function spatially and temporally for fine collaboration to
648 maintain lipid homeostasis in *Chlamydomonas* (Fig. 8). CrLACS1 and CrLACS2,
649 residing in the ER and/or LDs, function in activating fatty acids to support the
650 biosynthesis of ER membrane lipids as well as of a basal level of TAG under favorable
651 growth conditions. Suppressing each of *CrLACS1* and *CrLACS2* would trigger
652 transcriptional up-regulation of the other one for compensation thus avoiding
653 disturbance in membrane lipid biosynthesis and cell growth. Once exposed to nutrient
654 depletion, e.g., N-deprivation, *CrLACS1* and *CrLACS2* are substantially induced to
655 provide enough acyl-CoAs for the ER-localized acyltransferases such as type II
656 diacylglycerol acyltransferases (DGTT1/2/3); the peroxisome-localized CrLACS3, on
657 the other hand, is maintained at a basal level to minimize fatty acid β -oxidation, thus
658 allowing massive accumulation of TAG in *Chlamydomonas*. CrLACS1 and CrLACS2
659 function redundantly yet both are indispensable for TAG synthesis, which is packed

660 into the ER-derived LDs for storage. During N-recovery, CrLACS3 is triggered to
661 express to a very high level and acts in concert with TAG/DAG lipases (e.g., LIP4 and
662 LIP1) and fatty acid β -oxidation enzymes (e.g., ACX2) to remobilize TAG for
663 supplying energy and carbon for cell division and algal growth.

664

665 **Biotechnological implications**

666 We here have demonstrated the functionality of CrLACSs: CrLACS1 and CrLACS2
667 are involved in providing acyl-CoAs for TAG synthesis, while *CrLACS3* provides acyl-
668 CoAs in peroxisomes to enter fatty acid β -oxidation. These three CrLACS proteins are
669 therefore promising targets of genetic engineering to manipulate TAG accumulation in
670 algae. *CrLACS3* suppression has led to an increase in TAG level in *Chlamydomonas*
671 (Fig. 5c), which together with overexpression of *CrLACS1* or *CrLACS2* would have the
672 potential to further promote TAG accumulation. For the time being, we have confirmed
673 that heterologous expression of *CrLACS1* or *CrLACS2* promoted both TFA and TAG
674 levels in yeast (Fig. S12). These two genes may be applied to oleaginous algae for TAG
675 enhancement, as is the case of *CzLACS2* expressed in *Nannochloropsis oceanica* (Wu
676 *et al.*, 2020). Moreover, CrLACS enzymes particularly CrLACS2 possess strong
677 activities on eicosapentaenoic acid (EPA), a high-value compound beneficial to human
678 health (Lupette & Benning, 2020), and the activity (Fig. 1c) reaches over 10-fold
679 greater than that of *CzLACSs* (Wu *et al.*, 2020). They can be overexpressed in *N.*
680 *oceanica*, together with the acyltransferases that have potent activities in incorporating
681 EPA into TAG (Liu *et al.*, 2016a; Mao *et al.*, 2019), for the purposes of not only
682 promoting TAG accumulation but also enriching EPA in TAG.

683

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689

690 **Author contributions**

691 FB carried out the main experiments and analyzed the data; LY performed algal
692 cultivation and lipid analysis; JS maintained algal strains and performed algal
693 cultivation; YL-B gave critical comments and revised the paper; JL conceived the
694 idea, designed the research, analyzed the data and wrote the paper.

695

696 **Data Availability Statement**

697 The authors confirm that the data supporting the findings of this study are available
698 within the article and its supplementary materials

For Peer Review

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931

932 **Figure legends**

933 **Fig. 1** Growth, lipid levels and transcriptional expression of *CrLACS* genes in
934 *Chlamydomonas* under various N status.

935 (a) Schematic illustration of three-stage culture conditions (sequentially N replete - NR,
936 N deprivation - ND, then N deprivation-then-recovery - NDR). (b) Cell numbers. (c)
937 TFA and TAG contents. (d) Transcript levels of *CrLACS1*, *CrLACS2* and *CrLACS3*
938 relative to the internal control gene *β -actin*, quantified by RT-qPCR. MM, minimal
939 medium without acetate. Data represent mean \pm SD ($n=3$).

940

941 **Fig. 2** Functional characterization of CrLACS1, CrLACS2 and CrLACS3.

942 (a) Growth of YB525 cells carrying the empty vector pYES2 or *CrLACS* genes fed on
943 various free fatty acids (FFAs). Yeast growth was recorded at OD 600 nm after 10 days
944 of cultivation. (b) SDS-PAGE separation of recombinant CrLACS1, CrLACS2 and
945 CrLACS3 proteins from *E. coli*. (c) *In vitro* enzymatic assay of CrLACS proteins on
946 various FFAs. Data in (a, c) represent mean \pm SD ($n=3$).

947

948 **Fig. 3** Molecular characterization of the insertional mutants *crlacs1-1* and *crlacs3-1*.

949 (a, b) Schematic map (a) and PCR validation (b) of the insertion site in *CrLACS1* for
950 *crlacs1-1*. (c) Transcriptional levels of *CrLACS1* in *crlacs1-1* relative to WT. (d, e)
951 Schematic map (d) and PCR validation (e) of the insertion site in *CrLACS3* for *crlacs3-*
952 *1*. (f) Transcriptional levels of *CrLACS3* in *crlacs3-1* relative to WT. Arrows in (a, d)
953 designate primers used for validating insertion of the cassette. Data in (c, f) represent
954 mean \pm SD ($n=3$).

955

956 **Fig. 4** Comparison of growth and lipids between WT and *crlacs1-1* cells under NR, ND
957 and NDR conditions.

958 (a) Cell number per mL. (b) TFA content. (c) TAG content. (d) Fluorescent microscopy
959 observation of Bodipy-stained algal cells (ND day 3). Green signals indicate lipid
960 droplets. (e) Percentage of remaining TAG under NDR conditions (relative to ND day

961 3). Data in (a-c, f) represent mean \pm SD ($n=3$). Asterisks indicate the significant
 962 difference (Student's *t*-test, $P<0.05^*$ or $P<0.01^{**}$).

963

964 **Fig. 5** Comparison of growth and lipids between WT and *crlacs3-1* cells under NR, ND
 965 and NDR conditions.

966 (a) Cell number per mL. (b) TFA content. (c) TAG content. (d) Fluorescent microscopy
 967 observation of Bodipy-stained algal cells (day 3 of NDR). Green signals indicate lipid
 968 droplets. (e) Percentage of remaining TAG under NDR conditions (relative to ND day
 969 3). Data in (a-c, f) represent mean \pm SD ($n=3$). Asterisks indicate the significant
 970 difference (Student's *t*-test, $P<0.05^*$ or $P<0.01^{**}$).

971

972 **Fig. 6** Contents of membrane lipid classes in WT and mutants under NR and ND
 973 conditions.

974 (a) NR day 3. (b) ND day 3. Data represent mean \pm SD ($n=3$). Asterisks indicate the
 975 significant difference (Student's *t*-test, $P<0.05^*$ or $P<0.01^{**}$).

976

977 **Fig. 7** Complementation of *crlacs1-1* and *crlacs3-1* strains.

978 (a) The transcript levels of *CrLACS1* (relative to β -actin) in WT, *crlacs1-1* and the
 979 complemented strains on day 3 of ND as determined by RT-qPCR. (b) TAG contents in
 980 WT, *crlacs1-1* and the complemented strains under NR, ND and NDR conditions. (c)
 981 Contents of polar lipids in WT, *crlacs1-1* and the complemented strains on day 3 of ND.
 982 (d) The transcript levels of *CrLACS3* (relative to β -actin) in WT, *crlacs3-1* and the
 983 complemented strains on day 3 of NDR as determined by RT-qPCR. (e) TAG contents
 984 in WT, *crlacs3-1* and the complemented strains under ND and NDR conditions. (f)
 985 Percentage of remaining TAG on day 3 of NDR (relative to ND day 3) in WT, *crlacs3-*
 986 *1* and the complemented strains. Data represent mean \pm SD ($n=3$). Different letters
 987 above the bars in each group indicate significant difference ($p<0.05$), based on one-way
 988 analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test.

989

990 **Fig. 8** Proposed model explaining that CrLACS1, CrLACS2 and CrLACS3 function
991 spatially and temporally to achieve lipid homeostasis for growth and energy storage in
992 Chlamydomonas.
993 Multiple organelles are involved in lipid metabolism, including the chloroplast, ER,
994 LDs and peroxisomes, etc. CrLACS1 and CrLACS2 reside in the ER and/or LDs, and
995 contribute to TAG biosynthesis, while CrLACS3 is localized in peroxisomes and
996 contributes to TAG degradation. Under NR condition, growth is active for cell
997 reproduction; acyls are allocated predominantly to chloroplast membrane lipids (CMLs)
998 and ER membrane lipids (EMLs), with a basal level of TAG. Under ND condition,
999 growth is arrested; membrane lipid remodeling and TAG biosynthesis are stimulated
1000 while TAG catabolism is minimized, leading to massive accumulation of TAG for
1001 energy and carbon storage. Suppression of *CrLACS1* and *CrLACS2* each impairs TAG
1002 accumulation with increased levels of CMLs particularly MGDG and DGDG. Exposing
1003 ND-treated cells to NDR condition, TAG biosynthesis is minimized while TAG
1004 catabolism is stimulated, resulting in TAG degradation to supply carbon and energy
1005 sources for cell re-growth. *CrLACS3* suppression severely impairs TAG degradation
1006 and thus the cell re-growth. The thickness of green arrows refer to acyl fluxes (not on
1007 scale).

1008

1009 **Short legends for supporting information**

1010 Additional Supporting Information may be found online in the Supporting Information
1011 at the end of the article.

1012 **Fig. S1** Schematic map for expression of *CrLACS* genes in yeast (a), *E coli* (b) and in
1013 Chlamydomonas (c).

1014 **Fig. S2** Phylogenetic analysis of Chlamydomonas LACSs and their homologs from
1015 other organisms.

1016 **Fig. S3** Characterization of the insertional mutant *crlacs1-2*.

1017 **Fig. S4** Molecular confirmation of the insertional mutant of *crlacs2-1*.

1018 **Fig. S5** Comparison of dry weight per mL and per 10^6 cells between WT and mutants

1019 under ND and NDR conditions.

1020 **Fig. S6** Fatty acid composition of TFA and TAG of WT and *crlacs1-1* under NR, ND
1021 and NDR conditions.

1022 **Fig. S7** Comparison of growth and lipids between WT and *crlacs2-1* cells under NR,
1023 ND and NDR conditions.

1024 **Fig. S8** Fatty acid composition of TFA and TAG of WT and *crlacs2-1* under NR, ND
1025 and NDR conditions.

1026 **Fig. S9** Fatty acid composition of TFA and TAG of WT and *crlacs3-1* under NR, ND
1027 and NDR conditions.

1028 **Fig. S10** Global analysis of RNA-seq samples.

1029 **Fig. S11** Transcriptional expression of *CrLACS2* and *CrLACS3* in *crlacs1-1* (up) and
1030 of *CrLACS1* and *CrLACS3* in *crlacs2-1* (down) under NR and ND conditions.

1031 **Fig. S12** Heterologous expression of *CrLACS* genes in yeast promoted lipid synthesis.

1032 **Table S1** Primers used in the present study.

1033 **Table S2** Features of *CrLACS* genes.

1034 **Data S1** FPKM values of all samples.

1035 **Data S2** The RNA-Seq data for genes involved in selected biological pathways.