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1 **Short title**

2 *M. gaditana* Bubblegum acyl-CoA synthetase

3 **Title**

4 Characterization of the Bubblegum acyl-CoA synthetase of *Microchloropsis gaditana*

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23

24 **One sentence summary**

25 A heterokont Bubblegum acyl-CoA synthetase (ACSBG), or lipidosin, is essential in
26 *Microchloropsis (Nannochloropsis) gaditana* and thio-esterifies 16:1 and 18:3 fatty acids to
27 Coenzyme A *in vivo*.

28

29 **List of author contributions:**

30 E.B., L.M., S.L., M.B., L.P., A.A., S.Co. performed molecular biology experiments and
31 genetic and phenotypic studies; A.A.R. contributed to growth experiments in multiple devices
32 and contributed to phenotypic analyses; M.M and G.F provided specific expertise in
33 metabolic and physiologic analyses; F.R. and J.J. provided specific expertise in lipidomic
34 analyses; E.B., R.D. and S.Cr. performed bioinformatic and modeling analyses; E.B., L.F.,
35 F.L., S.Co., J.J. and E.M. participated to the conception of research plans; J.J and E.M.
36 conceived the project; all the authors contributed to the writing of the article.

37

38 **Keywords:**

39 Bubblegum Acyl-CoA synthase; lipidosin; *Nannochloropsis*, *Microchloropsis*;
40 eicosapentaenoic acid; very-long-chain polyunsaturated fatty acid; omega pathway

41

42

43 **Abstract**

44 The metabolic pathways of glycerolipids are well described in cells containing chloroplasts
45 limited by a two-membrane envelope but not in cells containing plastids limited by four
46 membranes, including heterokonts. Fatty acids (FAs) produced in the plastid, palmitic and
47 palmitoleic acids (16:0 and 16:1), are used in the cytosol for the synthesis of glycerolipids via
48 various routes, requiring multiple acyl-Coenzyme A (CoA) synthetases (ACS). Here, we
49 characterized an ACS of the Bubblegum subfamily in the photosynthetic eukaryote
50 *Microchloropsis gaditana*, an oleaginous heterokont used for the production of lipids for
51 multiple applications. Genome engineering with TALE-N allowed the generation of
52 MgACSBG point mutations, but no knockout was obtained. Point mutations triggered an
53 overall decrease of 16:1 in lipids, a specific increase of unsaturated 18-carbon acyls in
54 phosphatidylcholine and decrease of 20-carbon acyls in the betaine lipid diacylglyceryl-
55 trimethyl-homoserine. The profile of acyl-CoAs highlighted a decrease in 16:1-CoA and 18:3-
56 CoA. Structural modeling supported that mutations affect accessibility of FA to the
57 MgACSBG reaction site. Expression in yeast defective in acyl-CoA biosynthesis, further
58 confirmed that point mutations affect ACSBG activity. Altogether, this study supports a
59 critical role of heterokont MgACSBG in the production of 16:1-CoA and 18:3-CoA. In *M.*
60 *gaditana* mutants, the excess saturated and monounsaturated FAs were diverted to
61 triacylglycerol, thus suggesting strategies to improve the oil content in this microalga.

62

63

64 **Introduction**

65 In photosynthetic eukaryotes, the glycerolipid composition of subcellular membranes is
66 specific to each compartment; it results from the combination of biosynthetic pathways,
67 located mainly at the chloroplast and the endoplasmic reticulum (ER), and an intense
68 trafficking of lipid intermediates within the cell (Benning, 2008, 2009; Li-Beisson et al.,
69 2010; Boudiere et al., 2012; Horn and Benning, 2016; Li-Beisson et al., 2017; LaBrant et al.,
70 2018; Li-Beisson et al., 2019). Glycerolipids result from the combination of three types of
71 building blocks, *i.e.* a glycerol backbone originating from glycerol-3-phosphate (G3P, each
72 carbon being numbered as *sn*-1, *sn*-2 and *sn*-3), fatty acids (FAs) of different carbon chain
73 lengths and desaturation levels (indicated for instance as 18:1 for 18 carbons and 1 double
74 bond) and polar heads of various chemical structures (Supplemental Fig. S1).

75 The metabolic pathways are well described in cells containing chloroplasts limited by a two-
76 membrane envelope (so-called ‘primary plastids’ in Archaeplastida). A gap of knowledge
77 needs to be filled in cells containing plastids limited by four membranes (‘secondary plastids’
78 in various clades, including heterokonts) (Petroutsos et al., 2014; Li-Beisson et al., 2019).

79 In Archaeplastida, such as plants or green algae, *de novo* synthesis of FAs bound to acyl
80 carrier proteins (ACPs) occurs in the stroma of chloroplasts, generating acyl-ACPs, mainly
81 palmitoyl- and stearyl-ACP (16:0-ACP and 18:0-ACP), which can be desaturated by a
82 stromal acyl-ACP desaturase (AAD), producing 16:1-ACP and/or 18:1-ACP. Acyl-ACPs can
83 be directly used to esterify G3P at the inner envelope membrane of the plastid for the
84 production of precursors for the four major chloroplast glycerolipids, *i.e.* three classes of
85 glycolipids (monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol,
86 DGDG and sulfoquinovosyldiacylglycerol, SQDG) and one phosphoglycerolipid,
87 (phosphatidylglycerol, PG) (Li-Beisson et al., 2010; Boudiere et al., 2014). Chloroplasts also
88 export FAs and import glycerolipid precursors: acyl-ACPs can be converted into free FAs

89 (FFAs) by a chloroplast-specific thioesterase; FFAs then move to the cytosol, where they are
90 thioesterified to Co-enzyme A (CoA), thus forming acyl-CoAs (Li et al., 2015; Li et al., 2016;
91 Li-Beisson et al., 2017). This thioesterification is catalyzed by acyl-CoA synthetases (ACSs),
92 acting therefore in the control of FA fluxes in the cytosol, and their sorting toward different
93 end-products (Coleman et al., 2002; Mashek et al., 2006; Mashek et al., 2007). Acyl-CoAs are
94 acyl donors for the formation of the initial precursors for all membrane phosphoglycerolipids
95 in the ER, like phosphatidylcholine (PC) or phosphatidylethanolamine (PE), or for
96 triacylglycerol (TAG) stored in lipid droplets (Li-Beisson et al., 2010).

97 Superimposed to this glycerolipid biosynthetic network, FFAs can be ‘removed’ from a given
98 glycerolipid by specific lipases, releasing FFAs, and be replaced by the action of specific
99 acyl-CoA-dependent acyltransferases. This process is known as ‘acyl editing’ (Bates et al.,
100 2012; Hurlock et al., 2018; Menard et al., 2018). The pool of acyl-CoAs can be used for the
101 elongation of FFAs by specific elongases and the synthesis of other acylated molecules, such as
102 sterol-esters or acyl-proteins. Eventually, acyl-CoAs can be directed to catalytic organelles,
103 such as mitochondria or peroxisomes, to be specifically degraded by a process known as beta-
104 oxidation (Pei et al, 2003).

105 It is difficult to deduce the precise cellular function of ACSs, since they can be essential in the
106 activation of *de novo* synthesized FFAs as well as recycled ones, for a broad diversity of ‘acyl-
107 CoA-dependent’ purposes (Fig. 1). Some ACSs operate in more than one pathway (Coleman
108 et al., 2002; Mashek et al., 2006; Mashek et al., 2007). The function of each ACS depends
109 therefore on multiple parameters, including substrate specificity, temporal expression pattern,
110 location within the cell, interaction with other proteins, etc. Genetic impairment of ACS genes
111 appears therefore as a method of choice to address functionality.

112 By contrast with Archaeplastida, Heterokonta contain a plastid with four limiting membranes
113 (Fussy and Obornik, 2018). In models such as the diatom *Phaeodactylum tricornutum* or the

114 eustigmatophytes *Microchloropsis gaditana* and closely related species such as
115 *Nannochloropsis oceanica*, the two innermost membranes of the secondary plastid are
116 considered homologous to the two-membrane envelope of plant chloroplasts (the inner and
117 outer envelope membranes, IEM and OEM respectively). The *outermost membrane*, the
118 epiplastid membrane (EpM), is continuous with the outer nuclear envelope membrane
119 connected to the ER. The periplastid membrane (PpM) located between the EpM and the
120 OEM is of a still unresolved origin (Flori et al., 2016; Cavalier-Smith, 2018). The glycerolipid
121 biosynthetic scheme seems to follow some rules deciphered in plants, i.e. (1) a *de novo*
122 synthesis of FAs up to C16 in the stroma, generating 16:0-ACP, (2) the presence of enzymes
123 involved in MGDG, DGDG and SQDG biosynthesis probably located in plastid membranes
124 and (3) the presence of enzymes involved in phospholipid synthesis, such as PC and PE,
125 probably located in the ER (Petroutsos et al., 2014; Abida et al., 2015; Dolch and Marechal,
126 2015; Dolch et al., 2017). Photosynthetic heterokonts contain an additional glycerolipid class,
127 the betaine lipids, whose synthesis is usually associated with the ER. In *P. tricornutum* the
128 betaine lipid is diacylglyceryl-3-O-2'-(hydroxymethyl)-(N,N,N-trimethyl)- β -alanine (DGTA)
129 (Abida et al., 2015), whereas *M. gaditana* or *N. oceanica* contain diacylglyceryl-3-O-4'-
130 (N,N,N-trimethyl)-homoserine (DGTS) (Alboresi et al., 2016; Dolch et al., 2017; Murakami
131 et al., 2018).

132 Besides these general features, essential differences make it extremely challenging to transfer
133 knowledge acquired in plant or green algal models to Heterokonta:

- 134 - Firstly, after *de novo* synthesis in the plastid, 16:0 is exported to the cytosol, where it
135 is thioesterified into 16:0-CoA. This 16:0-CoA is the initial substrate for a series of
136 elongations and desaturations forming at first 18:0-CoA and ending up with very-long-
137 chain polyunsaturated fatty acids (VLC-PUFAs) in the ER, such as eicosapentaenoic
138 acid (EPA or 20:5) (Dolch et al., 2017). Since elongations operate on acyl-CoAs,

139 whereas desaturations occur on acyl-glycerolipids, the 16:0-to-20:5 route requires five
140 classes of enzymes, i.e. specific ACSs to supply elongases and acyl-transferases with
141 activated FAs, desaturases acting on a “glycerolipidic platforms” harboring the acyl-
142 group, where double bonds are added, and lipases A (Fig. 1). In *P. tricornutum* and *M.*
143 *gaditana*, based on the detection of 16:0-to-20:5 intermediates (mainly 18:0, 18:1,
144 18:2, 18:3, 20:3 and 20:4), the most likely glycerolipid platforms for 20:5 biosynthesis
145 are PC and the betaine lipid (Abida et al., 2015; Dolch et al., 2017; Sayanova et al.,
146 2017).

147 - Secondly, 16:0-ACP is presumed to be desaturated into 16:1-ACP in the plastid.
148 Palmitoleic acid is exported to the cytosol, where it is thioesterified into 16:1-CoA.
149 Palmitoleoyl-CoA is the preferred substrate for one of the four lysophosphatidic
150 acyltransferases occurring in *N. oceanica*, i.e. LPAT1, residing in the ER and adding
151 16:1 at position *sn*-2 of membrane glycerolipids, mainly PC and DGTS (Nobusawa et
152 al., 2017).

153 - Eventually, DGTS, MGDG and DGDG are marked by an extremely high content in
154 20:5 (Botte et al., 2011; Abida et al., 2013; Alboresi et al., 2016; Dolch et al., 2017;
155 Murakami et al., 2018). Genetic knock out of enzymes producing DGTS in *N.*
156 *oceanica* showed that this betaine lipid was a pool of 20:5 in the cytosol, where 20:5 is
157 actually synthesized (Murakami et al., 2018). To accumulate into MGDG and DGDG,
158 20:5 needs to relocate from the ER to the plastid, a process unlikely to be homolog of
159 the “eukaryotic pathway” described in Arabidopsis (*Arabidopsis thaliana*). This
160 import of 20:5 to the plastid of heterokonts has been termed the “omega pathway”
161 (Petroutsos et al., 2014; Dolch et al., 2017). Based on the genetic knock out of the
162 elongase *Δ0-elo1* in *M. gaditana*, the omega pathway seems to operate via a large-
163 scale multi-enzymatic and multi-site channeling machinery, starting from the initial

164 elongation of 16:0-CoA into 18:0-CoA by a Δ^0 -ELO isoform in the ER, and ending by
165 the formation of 20:5 and its transfer to MGDG in the plastid (Dolch et al., 2017).

166 In heterokonts, ACS isoforms are therefore expected to operate in FA fluxes at all these steps.
167 We have focused our study on *M. gaditana*, previously known as *Nannochloropsis gaditana*
168 (Fawley et al., 2015). *Microchloropsis* and *Nannochloropsis* are oleaginous microalgae,
169 producing high levels of TAG and representing therefore a promising feedstock for biofuel
170 and green chemistry, motivating important efforts into optimizing strains and culture
171 conditions (Van Vooren et al., 2012; Camacho-Rodriguez et al., 2013; Chen et al., 2013;
172 Camacho-Rodriguez et al., 2014; Ma et al., 2014; Camacho-Rodriguez et al., 2015; Meng et
173 al., 2015) and developing genetic engineering methods (Radakovits et al., 2012; Vieler et al.,
174 2012; Iwai et al., 2015; Kang et al., 2015; Dolch et al., 2017; Poliner et al., 2018). *M.*
175 *gaditana* is a model where C18 desaturation occurs mainly on PC, whereas C20 desaturations
176 occur mainly on DGTS and PE (Alboresi et al., 2016). To our knowledge, no genetic study
177 has focused on *ACS* genes in heterokonts. We addressed the role of an ACS isoform in *M.*
178 *gaditana* belonging to the Bubblegum subfamily (ACSBG) (Min and Benzer, 1999; Steinberg
179 et al., 2000; Pei et al., 2003; Mashek et al., 2007; Song et al., 2007; Lopes-Marques et al.,
180 2018) by genome engineering with tailored TALE-N.

181

182 **Results and Discussion**

183 **Identification of an Acyl-CoA synthetase of the « Bubblegum » subfamily (ACSBG) in**
184 ***M. gaditana***

185 We sought ACS genes that could be distinct between Heterokonta and Archaeplastida. The
186 genome of *M. gaditana* contains six genes annotated as Acyl-CoA synthetases (*ACS*) or Acyl-
187 CoA ligases (*ACL*): Naga_100014g59; Naga_100012g66; Naga_101051g1; Naga_100047g8;
188 Naga_100649g1 and Naga_100035g43
189 (http://protists.ensembl.org/Nannochloropsis_gaditana_gca_000569095/). The genome of the
190 model diatom *P. tricornutum* contains five putative *ACS* genes: Phatr3_J20143 (annotated as
191 *ACS1*), Phatr3_J12420 (*ACS2*), Phatr3_J54151 (*ACS3*), Phatr3_J45510 (*ACS4*) and
192 Phatr3_J17720 (*ACL1*) (http://protists.ensembl.org/Phaeodactylum_tricornutum). In the
193 current state of knowledge, it is not possible to predict the specificity of a given ACS
194 sequence for FAs.

195 Based on protein sequences, subfamilies of eukaryote ACSs have been defined (Steinberg et
196 al., 2000; Watkins et al., 2007). One subfamily corresponds to the “Bubblegum” Acyl-CoA
197 synthetases (ACSBG), named after the bubbly appearance of the first optic ganglion induced
198 by the mutation of the corresponding gene in the fruit fly *Drosophila melanogaster*, leading to
199 a neurodegeneration and elevated level of VLC-FAs (Min and Benzer, 1999). ACSBGs are
200 also called “lipidosins” (Song et al., 2007). ACSBGs have been mainly studied in Metazoa,
201 conserved from invertebrates to vertebrates (Lopes-Marques et al., 2018). The impairment of
202 *ACSBG* in *Drosophila* or mice leads to an increase of VLC-PUFAs, however the beta-
203 oxidation of VLC-PUFAs that could partly explain this increase seems not always affected in
204 corresponding *acsbg* mutants (Min and Benzer, 1999; Steinberg et al., 2000; Song et al.,
205 2007). The human (*Homo sapiens*) *ACSBG1* gene is expressed primarily in the brain
206 (Steinberg et al., 2000). Brains have higher concentrations of VLC-FAs than other tissues,

207 including VLC-PUFAs like 20:5 and 22:6; these VLC-FAs are components of complex lipids
208 such as gangliosides, cerebroside, sulfatides, sphingomyelin and other phospholipids, all
209 kinds of lipids whose synthesis could also rely on ACSBG (Steinberg et al., 2000). The
210 specific expression of *ACSBG* gene in animal brains as well as biochemical studies show that
211 ACSBG proteins are involved in the activation of PUFAs and VLC-PUFAs, with chain
212 lengths from 16 to at least 24 carbons (Steinberg et al., 2000). Concerning the two genes in
213 human, although *ACSBG1* was initially thought to activate VLC-FAs based on overexpression
214 studies (Steinberg et al., 2000), investigation of the endogenous enzyme using RNA
215 interference highlighted a high specificity for C16:0 (Pei et al., 2003). By contrast, *ACSBG2*
216 preferentially activates C18:1 and C18:2 acids (Watkins et al., 2007). In chicken, a single
217 nucleotide polymorphism in the *ACSBG2* gene is associated with abdominal fat weight and
218 percentage (Claire D'Andre et al., 2013). ACSBG proteins contain conserved domains, among
219 which motif II is possibly involved in FA-binding (Moriya-Sato et al., 2000; Steinberg et al.,
220 2000; Watkins et al., 2007; Lopes-Marques et al., 2018). Based on the conservation of motif
221 II (Fig. 2), we identified ACS isoforms of the bubblegum type in *M. gaditana* (*MgACSBG*;
222 Naga_100014g59) and *P. tricornutum* (annotated as *ACS4* and called here *PtACSBG*;
223 Phatr3_J45510). *MgACSBG* and *PtACSBG* share five conserved ACSBG motifs (Lopes-
224 Marques et al., 2018), further confirming their structural identification as ACSBG
225 (Supplemental Fig. S2).

226 We compared ACS sequences found in *M. gaditana* and *P. tricornutum* with sequences of
227 previously characterized ACSBG in animals, *i.e.* *D. melanogaster* (*DmACSBGa*,
228 NP_524698; *DmACSBGc*, NP_001285923); *Homo sapiens* (*HsACBG1*, Q96GR; *HsACBG2*,
229 Q5FVE4); *Mus musculus* (*MmACSBG1*, NP_444408); *Gallus gallus* (*GgACSBG1*, F1NLD6;
230 *GgACSBG2*, XP_015155301). Our Bayesian phylogenetic analysis showed a robustly
231 supported clade (posterior probability 1.00) grouping *M. gaditana* and *P. tricornutum*

232 ACSBG with metazoan bubblegum/lipidosin sequences. The other algal ACS sequences
233 grouped in separate clades (Fig. 3).

234 Using the MgACSBG sequence as query, and based on (1) similarity search using BlastP,
235 with a Blosum 62 similarity matrix, default parameters and an e-value threshold of 1×10^{-10} ,
236 and (2) detection of a conserved motif II, we could identify ACSBG homologues in both
237 photosynthetic (e.g. *Ectocarpus*, CBJ33608 or *Fistulifera*, GAX09496) and non-
238 photosynthetic (e.g. *Pithyium*, GAX99969 or *Phytophthora*, XP_008898610) heterokonts. By
239 contrast, we could not identify any sequence in Arabidopsis nor in any plant (Embryophyta).
240 Likewise, no ACSBG homologue could be found in the green alga model *Chlamydomonas*
241 *reinhardtii*. Nevertheless, some green algae, such as *Ostreococcus tauri* or *Micromonas*
242 *pusilla*, contained ACSBG genes, i.e. XP_003083453 and XP_003061097, respectively. Since
243 *Ostreococcus* differs from *Chlamydomonas* by its ability to produce high levels of VLC-
244 PUFAs up to 22:6 (Degraeve-Guilbault et al., 2017), the presence of an ACSBG might be
245 related to VLC-PUFA fluxes within the cell. We addressed the function of this class of
246 heterokont ACSBG in *M. gaditana*.

247

248 **TALE-N generation of *M. gaditana acsbg* mutants**

249 We sought to introduce mutations in the *MgACSBG* gene using a specifically designed
250 transcription activator-like effector nuclease (TALE-N), a method with high target binding
251 specificity compared to other gene-editing systems (Malzahn et al., 2017) and with the
252 possibility to generate different types of mutations, ranging from in-frame residue
253 substitutions to gene interruption and knock out (Ma et al., 2016). The target site for TALE-N
254 mutation in *MgACSBG* was selected by comparison with the whole genome to avoid multiple
255 binding of the nuclease. The TALE-N target site was located at the beginning of the coding
256 sequence to allow the generation of incomplete and inactive proteins. This region encodes a

257 hydrophobic domain of the protein, which might be important for the correct folding of the
258 protein, but distinct from ACSBG conserved motifs. Sequences coding for the two TALE-N
259 binding subunits (Tal2Ng-L and Talg2Ng-R) were designed as described in the Methods
260 section, to position the FokI DNA cleavage domain at the target site (Fig. 4, A), and they were
261 cloned in two independent vectors, pCT61 and pCT62, respectively. Following *M. gaditana*
262 co-transformation with pCT61 and pCT62 and selection on zeocin, obtained colonies were
263 firstly analyzed by treatment of PCR amplified target locus with a T7 endonuclease to detect
264 cleaved DNA at mismatched positions and assess the occurrence of genome editing. To define
265 precisely how the TALE-N modified the target DNA site, cells from T7-positive colonies
266 were sub-cloned onto a new selective plate to separate cells harboring the mutation from those
267 containing wild-type (WT)-like sequences.

268 Seven T7-positive lines were obtained from multiple independent co-transformations with the
269 pCT61 and pCT62 plasmids. No disruption of the *MgACSBG* full length coding sequence
270 could be obtained, suggesting that knockout (KO) might be lethal. Only nucleotidic
271 modifications leading to in-frame amino acid substitutions could be obtained (Fig. 4, A). In
272 some of the *M. gaditana* transformed lines, e.g. MgACSBG#5, MgACSBG#31 and
273 MgACSBG#40, TALE-N activity led to in-frame insertions/deletions and introduction of
274 non-synonymous codons, leading to point mutant proteins (Fig. 4, B).

275 ***In vivo impact of MgACSBG#5 and MgACSBG#31 in-frame mutations on glycerolipid*** 276 ***profiles***

277 We focused our phenotype analyses on two in-frame mutations, MgACSBG#5 and
278 MgACSBG#31. Cells were cultivated in parallel in a nutrient-rich liquid medium, using a
279 Multicultivator photobioreactor, supplied with CO₂ as described in the Methods section.
280 Control strains consisted of untransformed cells (wild type, WT) and cells transformed with
281 an empty vector (EV). Experiments were performed in duplicate for each line and were

282 repeated to obtain data from independent replicates ($n = 4$). The MgACSBG#5 and
283 MgACSBG#31 lines showed a slower growth compared to WT and EV strains, monitored by
284 cell counting at day 3, 7 and 12 following inoculation (D3, D7 and D12, respectively) (Fig. 5,
285 A). Compared to WT and EV strains, the total FA content per cell was higher in the
286 MgACSBG#31 mutant in the early stage of cultivation (D3) (Fig. 5, B), reflecting a higher
287 content in TAG (Fig. 5, C). WT and EV strains showed an accumulation of TAG at D7 and
288 D12 induced by the shortage of nutrients in the medium, as described earlier (Simionato et al.,
289 2013; Alboresi et al., 2016) (Fig. 5, C). MgACSBG#5 and MgACSBG#31 mutants also
290 showed increased TAG content at D7 and D12, but the difference with WT and EV strains
291 was less pronounced, probably due to the nitrogen starvation.

292 We focused our comparison of MgACSBG#5 and MgACSBG#31 with WT and EV cells at
293 D3, when no starvation of nutrients occurs. The total FA profile of mutant lines showed an
294 increase in 16:0 proportion balanced by a slight decrease in 16:1 in MgACSBG#31 and a
295 significant decrease of 20:5 in one of the mutants (Fig. 5, D). The glycerolipid profile
296 highlighted an increase in TAG and little change in the level of other glycerolipid classes
297 (Fig. 5, E). Since in *M. gaditana* TAG are 16:0-rich and 20:5-poor (Simionato et al., 2013;
298 Alboresi et al., 2016), one could consider that the global increase in 16:0 and the decrease in
299 20:5 in the mutant lines might simply reflect the accumulation of TAG. We addressed this
300 question by comparing the FA profile of each glycerolipid class at D3.

301 The comparison of FA profiles in glycerolipid classes in the ACSBG mutants with WT or EV
302 strains showed a striking impact on PC, DGTS and PE (Fig. 6, A-C), synthesized in the ER of
303 *M. gaditana*, and on two of the plastid lipids, MGDG and DGDG (Fig. 6, F-G), whereas little
304 or no significant change could be observed on PG and SQDG (Fig. 6, D-E). In LC-MSMS
305 analyses, m/z spectra correspond to diacyls indicated by the sum of carbons and the number

306 of double bonds (Alboresi et al., 2016), e.g. in PC, 34-2 corresponds to the major molecular
307 species 16:0/18:2.

308 We first noticed in the MgACSBG#5 and MgACSBG#31 mutants a decrease of molecular
309 species containing a 16:1 at sn-2 position in PC (e.g. 16:0/16:1; 16:1/16:1) and DGTS (e.g.
310 14:0/16:1; 16:1/16:1) (Fig. 6, A, B and C). This could reflect a role of ACSBG in producing
311 16:1-CoA for the *de novo* synthesis of lipids at the ER, reminiscent of the effect of the
312 knockout of LPAT in the closely related species *N. oceanica*, an enzyme shown to transfer
313 16:1 at the sn-2 position of these lipids (Nobusawa et al., 2017).

314 These lipids are presumed to also serve as “platforms” for the production of 20:5, *i.e.* PC from
315 16:0 to 18:3 and DGTS from 20:3 to 20:5. This role is not fully restricted to PC and DGTS: it
316 was shown in *N. oceanica* that mutants lacking DGTS lead to the accumulation of 18:1, 18:2
317 and 20:4 in PC, consistently with an accumulation of 20:5 precursors in this lipid, and more
318 20:4 and 20:5 in PE, consistently with PE acting as a secondary platform for the final steps of
319 20:5 production (Murakami et al., 2018). Here, in MgACSBG#5 and MgACSBG#31, PC
320 molecular species containing 18:1 and 18:2 (16:0/18:1; 16:0/18:2; 16:1/18:2) were enriched,
321 corresponding to an accumulation of 18:3 precursors (Fig. 6, A and Supplemental Table S1).

322 We had a closer look at PC acyl profiles and could detect 18:3 only in MgACSBG#5 and
323 MgACSBG#31 (16:1/18:3, 18:2-18:3 and 18:1/18:3; Supplemental Table S1). In these
324 mutants, DGTS had more diacyls with 16:0 and 18:1 (16:0/16:0; 18:1/16:0; 20:4/16:0;
325 20:5/16:0) and fewer diacyls containing only 20-carbon FAs (20:4/20:4; 20:5/20:4; 20:5/20:5)
326 (Fig. 6, B). PE was also altered, containing less 20:5 (16:1/20:5; 20:5/20:5) and higher levels
327 of 20:3 and 20:4 (20:4/20:3; 20:5/20:5) (Fig. 6, C). In the MgACSBG#5 and MgACSBG#31
328 mutants, 18-carbon precursors of 18:3 accumulated in PC (18:0; 18:1; 18:2 and 18:3), and
329 20:5 proportions decreased in DGTS and PE. This could reflect a role of ACSBG in
330 producing 18:3-CoA in the pathway producing 20:5 in DGTS and to some extent in PE.

331 The profile of MGDG in the MgACSBG#5 and MgACSBG#31 mutants is characterized, like
332 in the WT, by a major molecular species with a m/z corresponding to 34 carbons and 5 double
333 bonds (34-5 or 20:5/14:0). Little change was observed, besides a decrease of molecular
334 species enriched in 16:1 (16:1/16:0; 16:1/16:1) and a slight increase in 20:5-rich molecular
335 species (20:5/16:0; 20:5/20:5) (Fig. 6, F). DGDG showed the same trend but with a higher
336 magnitude, *i.e.* a dramatic decrease in the proportion of 16:1 (16:1/16:0; 16:1/16:1)
337 compensated by an increase in 20:5 (20:5/14:0, 20:5/16:0; 20:5/16:1) (Fig. 6, G). Since the
338 amount of plastid glycerolipids was either unchanged or slightly decreased in the mutants, the
339 observed phenotype in the MgACSBG#5 and MgACSBG#31 mutants reflected a depletion of
340 16-carbon FAs, mainly 16:1, partly compensated by imported 20:5.

341 The profile of TAG in the MgACSBG#5 and MgACSBG#31 mutants was also different from
342 that in the WT and EV lines. It was globally characterized by a decrease in 16:1-rich
343 molecular species (14:1/16:1/16:1; 16:1/16:1/16:0; 16:1/16:1/16:1; 16:1/18:1/16:1) and a
344 higher level of 16:0-rich molecular species (16:0/16:0/16:1; 16:0/16:0/16:0) as well as a
345 noticeable increase in 18:0 and 18:1 (16:1/16:0/18:0 and 16:1/16:0/18:1) (Fig. 6, H).

346 Altogether, these data indicate that a point mutation in the MgACSBG sequence leads to a
347 modification of FA partitioning within *M. gaditana* cells, altering two pathways, one being
348 the incorporation of 16:1 at the sn-2 position of ER glycerolipids via the LPAT1 pathway
349 dissected in *N. oceanica* (Nobusawa et al., 2017), another being the 16:0-to-20:5
350 elongation/desaturation process occurring on the PC and DGTS platforms, both leading to an
351 accumulation of 16:0 in ER lipids and TAG and an exhaustion of 16-carbon from plastid
352 glycerolipids.

353 The lack of KO mutant after TALE-N genetic mutation of MgACSBG suggests that this acyl-
354 CoA transferase operating in two key processes is critical. A possible interpretation is shown
355 in Figure 7, following FA biosynthesis in the plastid (Fig. 7, A), with a first role for ACSBG

356 in the production of 16:1-CoA in the cytosol (Fig. 7, B), prior to incorporation of 16:1 in ER
357 lipids via the LPAT1 pathway (Nobusawa et al., 2017) (Fig. 7, C). 16:0-CoA also entered the
358 16:0-to-20:5 channeling process, following a first elongation via a $\Delta 0$ -ELO producing 18:0-
359 CoA (Fig. 7, D). A second role for ACSBG in the production of 18:3-CoA was also deduced
360 from the mutants' phenotype in PC, DGTS and PE (Fig. 7, E-F). As a result of ACSBG
361 impairment, an over-accumulation of 16:0-CoA was expected and observed (Fig. 7, G). An
362 important assumption for this model is that a positive feedback is exerted on the plastid,
363 exporting most of its FAs, and explaining that the plastid is depleted of its 16-carbon FAs and
364 that the omega pathway is activated to provide sufficient 20:5 compensating the lack of 16-
365 carbon substrates in plastid glycerolipids (Fig. 7, H). In our scheme, the omega pathway
366 appears important for the synthesis of both MGDG and DGDG. The absence of any viable
367 knock-out mutant indicates that no other ACS isoforms from *M. gaditana* could apparently
368 compensate a lack of functional MgACSBG.

369 ***Impact of MgACSBG#5 and MgACSBG#31 mutations on acyl-CoA profiles***

370 Acyl-CoAs from WT, EV and mutant cells grown in nutrient replete condition were extracted
371 and sent to Rothamsted Research, UK, for a targeted analysis of chain lengths from 10 to 20
372 carbons (using 10:0; 12:0; 14:0; 16:0; 16:1; 16:3; 18:0; 18:1; 18:2; 18:3; 18:4; 18:5; 20:0;
373 20:3; 20:4 and 20:5 standards), as described in the Methods section (Fig. 8). The level of only
374 three acyl-CoAs showed a significant variation in mutants compared to WT, *i.e.* an increase in
375 16:0-CoA and a decrease in 16:1-CoA and 18:3-CoA. This analysis is consistent with a dual
376 role of ACSBG in the activation of 16:1 and 18:3 into 16:1-CoA and 18:3-CoA. We sought to
377 better comprehend the molecular impact of the mutation introduced in the MgACSBG
378 protein.

379 ***Molecular impact of MgACSBG#5 and MgACSBG#31 mutations on the protein structure.***

380 The MgACSBG sequence contains 649 amino acids and is predicted to be soluble with a MW
381 of 71,056 Da. We sought to construct a 3D structural model that could in part provide clues to
382 understand the effects of the mutations at the level of the IGF domain (a triad defined by
383 isoleucine 96, glycine 97 and phenylalanine 98). It seemed essential to propose the
384 positioning of a FA chain in the model, i.e. α -linolenic acid (18:3; C₁₈H₃₀O₂ *cis*- Δ 9,12,15), as
385 a substrate for MgACSBG deduced from phenotypic analyses. The search of conserved acyl-
386 CoA synthetase motifs was refined *via* the online servers MOTIF (Kanehisa et al., 2002) and
387 PROSITE (Sigrist et al., 2013), which identified an AMP binding motif, while Superfamily
388 (Wilson et al., 2009) confirmed that MgACSBG belonged to an Acetyl-CoA synthetase-like
389 family. The Porter predicting method (Mirabello and Pollastri, 2013) found 44% coil, 37%
390 helix and 19% extended conformation while Predictprotein (Yachdav et al., 2014) gave the
391 secondary structure prediction shown in Supplemental Fig. S3.

392 The sequence alignment of MgACSBG with 4 proteins used as templates in our models (see
393 below) is shown in Supplemental Fig. S4. The most clearly conserved motif YTSGTTGPPK
394 (residues 216-225), identified as the AMP binding motif, was requalified as playing a
395 fundamental role in ACS catalytic activity (Gulick et al., 2003; Lopes-Marques et al., 2018).
396 The long sequence SITGRIKELIITAGGENIPPVLIE (residues 492-515), or “motif II”,
397 contains a part of the “FACS motif” (fatty acyl CoA synthetase signature motif) (Black et al.,
398 1997) and is supposed to be involved in acyl chain length specificity. In ACSBGs, a so-called
399 “motif III” (F/YG-SE) (residues 409-413) has been proposed as being involved in AMP
400 binding, whereas “Motif IV” (LPLSH) (residues 259-263) could be involved in CoA binding
401 (Lopes-Marques et al., 2018).

402 No structure is currently available for an ACSBG in public databases. The sequence was first
403 submitted to Blast (Altschul et al., 1990) to find sequence alignments with proteins of known
404 3D structure. The first hit was referenced 1ULT in the Protein Data Bank (PDB, (Burley et

405 al., 2019)) and corresponds to a long chain fatty acyl-CoA synthetase homodimer from
406 *Thermus thermophilus* (Hisanaga et al., 2004), with 27% sequence identity and 40%
407 homology. Then the sequence was submitted to several online prediction servers (Robetta
408 (Song et al., 2013) ; RaptorX (Kallberg et al., 2012); Swiss-Model (Waterhouse et al., 2018);
409 PhyRe2 (Kelley et al., 2015) ; I-Tasser (Zhang, 2009); PsiPred (Buchan et al., 2013) and
410 PS(2) (Huang et al., 2015)). All methods failed to predict the full MgACSBG protein and
411 most of them found 1PG4 (acetyl-CoA synthetase from *Salmonella enterica*) (Gulick et al.,
412 2003), and 5MSC (the A domain of carboxylic acid reductase from *Nocardia iowensis*)
413 (Gahloth et al., 2017) as templates. Despite its low homology with MgACSBG, another acyl-
414 CoA synthetase with known 3D structure and for which both AMP and CoA are resolved was
415 chosen as template: 3EQ6, a human acyl-CoA synthetase medium-chain family member
416 (Kochan et al., 2009). The sequences of these four template proteins were aligned with
417 MgACSBG in Supplemental Fig. S4.

418 3D structural homology models of the full length MgACSBG protein were built with
419 Modeller (Sali and Blundell, 1993) using different association of template proteins: M1pg4
420 from 1PG4 alone, M3eq6 from 3EQ6 alone and two consensus models Mcons1 from 1PG4,
421 1ULT and 5MSC, and Mcons2 from all four protein templates (Supplemental Models 1 to 4).
422 The positioning of CoA and AMP in the MgACSBG models was based on their neighboring
423 residues in structure 1PG4 and 3EQ6, also identified in animal ACSBG2 (Steinberg et al.,
424 2000) (Supplemental Tables S2) as described in the Methods section. The positioning of 18:3
425 was placed inside the structures, with the carboxy-terminal “head” in the direction of AMP,
426 respecting its proximity with CoA and profiting of hydrophobic holes observed in the
427 structures.

428 Three models of MgACSBG, M1pg4, M3eq6 and Mcons1, have similar final energy and
429 correctly respect the restraints. From model Mcons1 of MgACSBG (Fig. 9), the proximity of

430 the IGF triad, mutated in this study, to the inserted 18:3 FA is clear. Their relative position is
431 globally the same in all models. Our modelling supports therefore the hypothesis that the
432 mutation could affect the accessibility of the FA to the reaction site rather than interfere with
433 the reaction itself through proximity with AMP or CoA.

434 ***MgACSBG but not MgACSBG#5 or MgACSBG#31 partially complement a yeast defective***
435 ***in acyl-CoA synthase function.***

436 We sought to decipher whether MgACSBG and its two variants could be active after
437 heterologous expression in the yeast *Saccharomyces cerevisiae* and complement the
438 phenotype of a *faa1Δfaa4Δ* double mutation. In yeast, acyl-CoA can originate from neo-
439 synthesis by a fatty acyl synthase (FAS) or by the activation of exogenous FAs by ACSs
440 (Black and DiRusso, 2007). Faa1 and Faa4 are the major enzymes involved in the formation
441 of long chain fatty acyl-CoA (Johnson et al., 1994). In normal growth condition, the
442 *faa1Δfaa4Δ* mutant does not show any growth defect. However, when yeast FAS is
443 inactivated by the addition of cerulenin, the mutant strain cannot grow, even in the presence
444 of exogenously supplied FAs, due to a low level of acyl-CoA synthetase activity (less than
445 5% of WT activity) (Johnson et al., 1994; Faergeman et al., 2001). MgACSBG,
446 MgACSBG#5 and MgACSBG#31 were expressed in the yeast *faa1Δfaa4Δ* mutant from a
447 high copy vector under the galactose inducible pGAL1 promoter. The WT and *faa1Δfaa4Δ*
448 strains transformed with the empty vector were used as controls. Control strains will be
449 henceforth referred to as ‘yeast WT’ and ‘*faa1Δfaa4Δ* mutant’, respectively, for simplicity.
450 All the strains were grown on synthetic media deprived of uracil (-U) and supplemented with
451 galactose 2% for 6 h to induce expression of the transgenes prior to dilution to $OD_{600} = 0.3$.
452 Growth curves were then performed at 30°C. All the strains used in the complementation
453 experiments were tested for expression of MgACSBG variants by immunoblot (Supplemental

454 Fig. S5). All strains presented an exponential growth in synthetic medium with galactose but
455 did not grow in the presence of 22.5 μ M cerulenin, as expected (Supplemental Fig. S5).

456 In a first set of experiments, we tested the ability of a series of FAs, namely 14:0, 16:0, 18:0,
457 18:1, 18:2, and 18:3, to rescue the growth of the yeast WT in presence of cerulenin. In our
458 conditions, 14:0 and 16:0 FAs were able to almost fully complement the growth defect
459 induced by cerulenin, whereas 18:0 and 18:1 only partially did, as previously observed by
460 others (Fig. 10, A) (Johnson et al., 1994; Johnson et al., 1994). Conversely, 18:2 and 18:3 FAs
461 were not able to rescue the growth of yeast WT in presence of cerulenin, likely because these
462 two FAs are not naturally present in yeast.

463 We next investigated the ability of MgACSBG native sequence to complement the
464 *faa1 Δ faa4 Δ* strain in presence of cerulenin with the same set of FAs previously tested. Three
465 independent clones were analyzed. The results presented in Figure 10, B show that
466 MgACSBG partially rescued the growth phenotype of the double mutant in presence of C14:0
467 and C16:0. Altogether, these results provide evidence that MgACSBG has indeed an acyl-
468 CoA synthetase activity, is able to use at least 14:0 or 16:0 but cannot provide more
469 information on the specificity for other FAs. MgACSBG-transformed yeast have the same
470 order of apparent preference for 14:0 and 16:0 as that observed in the wild-type yeast (Fig. 10,
471 A), thus indicating that this method is not suitable to address the question of the substrate
472 specificity of MgACSBG at the molecular level.

473 In the light of the results presented above, 14:0 and 16:0 were retained for further
474 investigation. The same experiments were performed with three independent clones
475 expressing MgACSBG#5 and MgACSBG#31 in a *faa1 Δ faa4 Δ* background in presence of
476 cerulenin and 14:0 or 16:0. Neither MgACSBG#5 nor MgACSBG#31 (Figs 10, C-D;
477 Supplemental Fig S5, C-E) were able to complement the growth phenotype of the mutant
478 strains in presence of 14:0 and 16:0, unlike MgACSBG. Altogether, these results indicate that

479 the TALE-N generated mutations interfered with the acyl-CoA synthetase activity of
480 MgACSBG, and that this decline in activity was related with the observed phenotype in in *M.*
481 *gaditana*.

482

483

484 **Conclusion and perspectives.**

485 ACS enzymes generate acyl-CoA pools at different locations of the cell, of different FA
486 profiles and for specific acylation reactions. They are therefore key actors of FA partitioning
487 in lipid metabolism. Unraveling an ACS specific role is made difficult by the possibility of
488 multiple isoforms to compensate a defective enzyme. In photosynthetic eukaryotes, ACS are
489 therefore expected to operate in important acyl fluxes, most importantly from the plastid to
490 the cytosol and vice versa. Heterokonta are marked by the production of VLC-PUFAs in the
491 cytosol, rapidly transferred to the secondary plastid by a channeled processed called the
492 omega pathway. ACS isoforms are expected to operate at ‘strategic’ levels in this metabolon,
493 in particular after release of 18:3-FFA following acyl desaturation on PC, to generate 18:3-
494 CoA for elongation into 20:3-CoA, precursor for further desaturations on DGTS and/or PE to
495 generate eicosapentaenoic acid, 20:5. Our functional analyses of the *M. gaditana* Bubblegum
496 ACS showed that this enzyme was likely vital, since no KO could be obtained, and that a
497 functional defect impaired two critical steps of acyl-lipid metabolism, the activation of
498 palmitoleic acid, generating 16:1-CoA used to acylate the sn-2 position of ER lipid, and of
499 linolenic acid, generating 18:3-CoA used as intermediate in the 16:0-to-20:5 synthetic
500 process. The obtained mutants using TALE-N editing still contain 20:5, probably because the
501 level of this VLC-PUFA cannot fall below a critical threshold necessary for *M. gaditana*
502 survival. Our results do not exclude that the Heterokont ACSBG also acts in other cellular
503 locations, for other purposes. In a recent study, the *P. tricornutum* homologue,
504 PtACSBG/ACS4, was found in the proteome of the lipid droplet (Lupette et al., 2019),
505 indicating a possible role in the acyl-CoA pool closely associated to this cytosolic organelle.
506 Future works will include the study of this enzyme’s location within *M. gaditana*, which is a
507 challenging task given the small size of this microalga. The search of protein partners is also
508 an important perspective, to evaluate the possible association with LPAT1 acting on ER lyso-

509 lipids, or other components of the machinery converting 16:0 into 20:5 (such as Δ^0 -ELOs,
510 ERAFAD9, ERAFAD12, a PC-specific phospholipase A, Δ^5 -ELOs, ERA Δ^5 FAD, ER ω^3 FAD, a
511 DGTS/PE-specific lipase A, and subunits of a still-to be identified carrier/transporter
512 importing a 20:5-precursor inside the secondary plastid), or any other protein component that
513 could further highlight the full range of function of this type ACS in a photosynthetic
514 eukaryote.

515 **Materials and methods**

516 ***MgACSBG phylogenetic analysis.***

517 All genes annotated as acyl-CoA synthetases or ligases from *Microchloropsis gaditana*
518 (Naga_100014g59, MgACSBG; Naga_100012g66; Naga_101051g1; Naga_100047g8;
519 Naga_100649g1 and Naga_100035g43) and *Phaeodactylum tricornutum* (Phatr3_J20143,
520 ACS1; Phatr3_J12420, ACS2; Phatr3_J54151, ACS3; Phatr3_J45510, PtACSBG or ACS4;
521 and Phatr3_J17720, ACL1) were retrieved from public databases,
522 http://protists.ensembl.org/Nannochloropsis_gaditana_gca_000569095/ and
523 http://protists.ensembl.org/Phaeodactylum_tricornutum, respectively. These sequences were
524 compared with ACSBG sequences from Metazoa, *i.e.* *Drosophila melanogaster*
525 (DmACSBGa, NP_524698; DmACSBGc, NP_001285923); *Homo sapiens* (HsACBG1,
526 Q96GR2; HsACBG2, Q5FVE4); *Mus musculus* (MmACSBG1, NP_444408) and *Gallus*
527 *gallus* (GgACSBG1, F1NLD6; GgACSBG2, XP_015155301). A phylogenetic tree was
528 reconstructed after a multiple alignment of sequences using the MUSCLE method (v3.8.31)
529 configured for high accuracy with default settings (Edgar, 2004), removal of gaps and poorly
530 aligned sequences using Gblock (Castresana, 2000). The tree was calculated using the
531 phylogeny.fr internet platform (Dereeper et al., 2008) using a Bayesian inference method
532 implemented in the MrBayes program (v3.2.6), with a number of substitution types fixed to 6,
533 a Poisson model for amino acid substitutions, four Markov chain Monte Carlo chains run for
534 10,000 generations, sampling every 10 generations, discard of the first 250 trees sampled and
535 a 50% majority rule consensus tree (Huelsenbeck and Ronquist, 2001; Ronquist et al., 2012).

536 ***Cultivation of M. gaditana cells***

537 *M. gaditana* Strain CCMP526 was used in all experiments. For nuclear transformation, cells
538 were grown under constant light in f/2 medium (Dolch et al., 2017) until they reached the late
539 exponential phase. All cultures were maintained on f/2 plates solidified with 1% agar, w/v,

540 under a 12:12 light/dark regime in presence (transformed strains) or absence (wild-type strain)
541 of the selective antibiotic zeocin ($7 \mu\text{g}\cdot\text{mL}^{-1}$). For phenotypic analyses, *M. gaditana* was
542 cultivated in artificial seawater (ESAW) using ten times enriched nitrogen and phosphate
543 sources ($5.49 \times 10^{-3} \text{ M NaNO}_3$ and $2.24 \times 10^{-4} \text{ NaH}_2\text{PO}_4$; called “10X ESAW”) (Dolch et al.,
544 2017). When grown in batch in 250 mL flasks, a volume of 50 mL liquid medium was
545 inoculated at $2.5 \times 10^6 \text{ cells}\cdot\text{mL}^{-1}$ and cultivation was achieved under gentle agitation (100
546 RPM) at 20°C on a 12:12 light ($60 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) / dark cycle. When grown with a
547 controlled CO_2 supply, a volume of 80 mL liquid medium was inoculated at 2.5×10^6
548 $\text{cells}\cdot\text{mL}^{-1}$ and cultivation was achieved in small photobioreactors (Multi-Cultivator MC 1000,
549 Photon Systems Instruments, Czech Republic) at 24°C , in continuous light (illumination at 60
550 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Culture mixing was achieved by gas bubbling in which CO_2 levels
551 were maintained constant at 0.5% as in air-lift photobioreactors. Precise and constant level of
552 CO_2 was supplied by a Gas Mixing System GMS 150 (Photon Systems Instruments, Czech
553 Republic) following manufacturer’s instructions. In a multicultivator system, growth was
554 conducted in nutrient replete medium until day 3, then transferred to same medium without
555 nitrogen to investigate possible impact on low nitrogen response. When needed, cells were
556 counted using a LUNA Automated Cell Counter following manufacturer's instructions. For
557 lipidomic analyses, cells were harvested by centrifugation at $3,500 \times g$ for 10 minutes. Cells
558 were then immediately frozen in liquid nitrogen and stored at -80°C , until use.

559 ***Design of vectors containing MgACSBG-specific TALE-N subunits***

560 Transcription activator-like effector nucleases (TALE-N) consisted of restriction enzymes
561 made of two subunits, called here Tal2Ng-R and Talg2Ng-L, engineered by fusing a TAL
562 effector DNA-binding domain to a FokI DNA cleavage domain. The DNA binding domain
563 contained a repeated highly conserved amino acid sequence with divergent amino acids,
564 referred to as the repeat variable diresidue (RVD). The Tal2Ng-L and Tal2Ng-R RVDs were

565 purchased from ThermoFisher GeneArt-TALs and synthesized into their commercial vector.
566 Tal2Ng-L subunit was designed following established guidelines (Sanjana et al., 2012) to
567 bind to the TCCTGCGCATGGTGCCATT sequence in the *MgACSB* gene
568 (Naga_100014g59), corresponding to RVD (T)-HD-HD-NG-NN-HD-NNHD-NI-NG-NN-
569 NN-NG-NN-HD-HD-NI-NG-NG, whereas the Tal2Ng-R subunit was designed to bind
570 specifically to TGGCAATGAGCCATTCGGG, corresponding to RVD (T)-NN-NN-HD-NI-
571 NI-NGNN-NI-NN-HD-HD-NI-NG-NG-HD-NN-NN-NN. In these RVD sequences, '(T)'
572 indicates that the first binding repeat is provided by the vector. Part of the Tal2Ng-L and
573 Tal2Ng-R commercial sequences (comprising RVDs) were subcloned in two distinct home-
574 designed vectors pCT5Ng and pCT6Ng to give pCT61 and pCT62, respectively. pCT5Ng is a
575 pET15b backbone which contains a first part with the bleomycin/zeomycin-resistance protein
576 from *Streptoalloteichus hindustanus* (ShBle) under the control of the UEP and with the fcpA
577 terminator and a second part with a codon-adapted nuclear localization sequence (NLS)
578 DYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAA (Sanjana et al., 2012), a
579 codon-adapted HA-tag, N-ter sequence of commercial TAL up to *AflIII* site, C-ter sequence of
580 commercial TAL from *XhoI* site under the control of the endogenous ubiquitin extension
581 promoter (UEP) and with the fcpA terminator. pCT6Ng is a pET28b backbone which contains
582 a first part with the bleomycin/zeomycin-resistance protein from *Streptoalloteichus*
583 *hindustanus* (ShBle) under the control of the UEP and with the fcpA terminator and a second
584 part with a codon-adapted nuclear localization sequence (NLS)
585 DYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAA (Sanjana et al., 2012), a
586 codon-adapted HA-tag, N-ter sequence of commercial TAL up to *AflIII* site, C-ter sequence of
587 commercial TAL from *XhoI* site under the control of the endogenous ubiquitin extension
588 promoter (UEP) and with the fcpA terminator. Codon optimization for expression in *M.*
589 *gaditana* was done using the Kazusa webtool (<http://www.kazusa.or.jp/codon/>).

590 ***Nuclear transformation of M. gaditana***

591 Plasmids pCT61 and pCT62 were linearized by digestion with ScaI and column-purified
592 using a NucleoSpin gel and polymerase chain reaction (PCR) clean-up kit (Macherey-Nagel)
593 following manufacturer's instructions. Two micrograms (one microgram TALE-N left subunit
594 plasmid + one microgram TALE-N right subunit plasmid) linearized plasmids were
595 electroporated into *M. gaditana* as previously described (Dolch et al., 2017). Transformed
596 lines were selected on f/2 plates containing 7 $\mu\text{g}\cdot\text{mL}^{-1}$ zeocin. Integration of both TALE-N
597 subunits was assessed by colony PCR with primers TAL-Nterm-Rev
598 (GCAGGTCGCTAAAAGAATCG) and TAL-HA Fw (CCCCGACTACGCTAGCG) for
599 TALEN left subunit and TAL-Nterm-Rev (GCAGGTCGCTAAAAGAATCG) and TAL-His
600 Fw (CACCACCACCACCACAGC) for TALEN right subunit.

601 ***T7 endonuclease test and sequencing of TALE-N genetic targets***

602 Following *M. gaditana* co-transformation with pCT61 and pCT62 and selection on zeocin,
603 obtained clones harboring both TALE-N subunits were firstly analyzed by treatment with a
604 T7 endonuclease (T7E1, New England Biolabs) to cleave DNA at mismatched positions and
605 assess occurrence of genome editing at the target locus. To this aim, a genomic fragment of
606 around 396 bp in the *MgACSB* gene (Naga_100014g59), where the FokI activity region is
607 placed asymmetrically from the 5' and 3' ends, was PCR-amplified from positive colonies,
608 using a proofreading polymerase. After electrophoresis of amplified DNA on a 1% agarose
609 gel, bands corresponding to the desired products were purified and quantified. For each clone,
610 1.3 μg of PCR product was treated with T7E1 (+) or left untreated (-). A non-transformed
611 wild-type (WT) colony was also included as a negative control. T7E1 reaction was performed
612 following manufacturer's instruction, with the following minor modifications. One microliter
613 of T7E1 enzyme was used in each reaction and incubation performed at 37°C for one hour.
614 Separation of obtained fragments on 2% agarose gels allowed the detection of mismatched

615 DNA and occurrence of mutations at target sites in some of the clones tested. Positive *M.*
616 *gaditana* colonies were transferred onto a new selective plate to separate cells harboring the
617 mutation from those that presented a WT-like sequence. Mutation at *MgACSBG* target sites
618 was confirmed by sequencing of the DNA at the target locus (Eurofins, France).

619 ***Glycerolipid analyses***

620 Glycerolipids were extracted from freeze-dried *M. gaditana* cells grown in 50 mL of indicated
621 medium. About 50 to 100×10^6 cells were required for each triplicate analysis. A freeze-dried
622 pellet was suspended in 4 mL of boiling ethanol for 5 minutes to prevent lipid degradation,
623 and lipids were extracted as described earlier (Simionato et al., 2013) by addition of 2 mL
624 methanol and 8 mL chloroform at room temperature. The mixture was saturated with argon
625 and stirred for 1 hour at room temperature. After filtration through glass wool, cell debris was
626 rinsed with 3 mL chloroform/methanol 2:1, v/v, and 5 mL of NaCl 1%, w/v, was then added
627 to the filtrate to initiate biphasic formation. The chloroform phase was collected and dried
628 under argon before solubilizing the lipid extract in 1 mL of chloroform. Total glycerolipids
629 were quantified based on their fatty acid (FA) content: in a 10 μ l aliquot fraction, a known
630 quantity of 15:0 was added and FAs were converted into FA methyl esters (FAME) by a 1
631 hour incubation in 3 mL 2.5% H₂SO₄ in pure methanol, v/v, at 100°C (Jouhet et al., 2003).
632 The reaction was stopped by addition of 3 mL water, and 3 mL hexane was added for phase
633 separation. After 20 minutes incubation, the hexane phase was transferred to a new tube.
634 FAMEs were extracted a second time via the addition, incubation and extraction of another 3
635 mL hexane. The combined 6 mL were argon-dried and re-suspended in 30 μ l hexane for gas
636 chromatography-flame ionization detector (GC-FID) (Perkin Elmer) analysis on a BPX70
637 (SGE) column. FAMEs were identified by comparison of their retention times with standards
638 (Sigma) and quantified by the surface peak method using 15:0 for calibration. Extraction and
639 quantification were performed with at least three biological replicates. Glycerolipids were

640 then analyzed and quantified by high pressure liquid chromatography-tandem mass
641 spectrometry (HPLC-MS/MS), with appropriate standard lipids. The lipid extracts
642 corresponding to 25 nmol of total fatty acids were dissolved in 100 μ L of
643 chloroform/methanol [2/1, (v/v)] containing 125 pmol of each internal standard. Internal
644 standards used were phosphatidylethanolamine (PE) 18:0-18:0 and diacylglycerol (DAG)
645 18:0-22:6 from Avanti Polar Lipid, and sulfoquinovosyldiacylglycerol (SQDG) 16:0-18:0
646 extracted from spinach (*Spinacia oleracea*) thylakoid (Deme et al., 2014) and hydrogenated
647 (Buseman et al., 2006). Lipids were then separated by HPLC and quantified by MS/MS. The
648 HPLC separation method was adapted from previously described procedure (Rainteau et al.,
649 2012). Lipid classes were separated using an Agilent 1200 HPLC system using a 150 mm \times 3
650 mm (length \times internal diameter) 5 μ m diol column (Macherey-Nagel), at 40°C. The mobile
651 phases consisted of hexane/isopropanol/water/1 M ammonium acetate, pH 5.3 [625/350/24/1,
652 (v/v/v/v)] (A) and isopropanol/water/1 M ammonium acetate, pH 5.3 [850/149/1, (v/v/v)] (B).
653 The injection volume was 20 μ L. After 5 min, the percentage of B was increased linearly
654 from 0% to 100% in 30 min and kept at 100% for 15 min. This elution sequence was followed
655 by a return to 100% A in 5 min and an equilibration for 20 min with 100% A before the next
656 injection, leading to a total runtime of 70 min. The flow rate of the mobile phase was 200
657 μ L/min. The distinct glycerophospholipid classes were eluted successively as a function of
658 the polar head group. Mass spectrometric analysis was performed on a 6460 triple quadrupole
659 mass spectrometer (Agilent) equipped with a Jet stream electrospray ion source under
660 following settings: drying gas heater at 260°C, drying gas flow at 13 L \cdot min⁻¹, sheath gas
661 heater at 300°C, sheath gas flow at 11 L \cdot min⁻¹, nebulizer pressure at 25 psi, capillary voltage
662 at \pm 5000 V and nozzle voltage at \pm 1,000 V. Nitrogen was used as collision gas. The
663 quadrupoles Q1 and Q3 were operated at widest and unit resolution respectively.
664 Phosphatidylcholine (PC) and diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS)

665 analyses were carried out in positive ion mode by scanning for precursors of m/z 184 and 236
666 respectively at a collision energy (CE) of 34 and 52 eV. SQDG analysis was carried out in
667 negative ion mode by scanning for precursors of m/z -225 at a CE of -56eV. PE,
668 phosphatidylinositol (PI), phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG)
669 and digalactosyldiacylglycerol (DGDG) measurements were performed in positive ion mode
670 by scanning for neutral losses of 141 Da, 277 Da, 189 Da, 179 Da and 341 Da at CEs of 20
671 eV, 12 eV, 16 eV, 8 eV and 8 eV, respectively. DAG and triacylglycerol (TAG) species were
672 identified and quantified by multiple reaction monitoring (MRM) as singly charged ions
673 $[M+NH_4]^+$ at a CE of 16 and 22 eV respectively. Quantification was done for each lipid
674 species by multiple reaction monitoring (MRM) with 50 ms dwell time with the various
675 transitions previously recorded. Mass spectra were processed using the MassHunter
676 Workstation software (Agilent) for identification and quantification of lipids. Lipid amounts
677 (pmol) were corrected for response differences between internal standards and endogenous
678 lipids as described previously (Jouhet et al., 2017).

679 *Acyl-CoA Analysis*

680 Freshly harvested cells cultivated in 10X ESAW (nutrient replete) medium were frozen in
681 liquid nitrogen, lyophilized and transferred to Rothamsted Research, UK for acyl-CoA
682 extraction and analysis (15 to 30 mg dry weight per sample). In brief, acyl-CoAs were
683 extracted using a bead beating method and analyzed using liquid chromatography coupled to
684 tandem mass spectrometry (LC-MS/MS) plus multireaction monitoring (MRM) in positive
685 ion mode. Acyl-CoA extraction efficiency was normalized to a 17:0 CoA (Sigma-Aldrich)
686 internal standard. All samples were immediately analyzed after extraction and were
687 maintained on a cooled stage prior to LC-MS/MS plus MRM analysis (using an ABSciex
688 4000 QTRAP device), as described earlier (Haynes et al., 2008) using an Agilent 1200 LC
689 system with a Agilent Eclipse XDB-C18 (3×100 mm; 3.5- μ m particle size) column. For the

690 purposes of identification and calibration, standard acyl-CoA esters with acyl chain lengths
691 from C10 to C20 (10:0; 12:0; 14:0; 16:0; 16:1; 16:3; 18:0; 18:1; 18:2; 18:3; 18:4; 18:5; 20:0;
692 20:3; 20:4; 20:5) were purchased from Sigma-Aldrich as free acids or lithium salts.

693 ***Modeling of MgACSBG structure bound to Coenzyme A, alpha-linolenic acid (18:3) and***
694 ***AMP***

695 Structure modeling was based on the identification of acyl-CoA synthetase conserved motifs,
696 secondary structure predictions and alignments with homologous sequences, for which protein
697 3D structures have been resolved and stored in the Protein Data Bank (PDB, (Burley et al.,
698 2019)). Motif predictions were performed using the online servers MOTIF (Kanehisa et al.,
699 2002), PROSITE (Sigrist et al., 2013) and Superfamily (Wilson et al., 2009). Secondary
700 structures were predicted using the Porter (Mirabello and Pollastri, 2013) and Predictprotein
701 (Yachdav et al., 2014) online tools. Search for homologous sequences was performed using
702 Blast (Altschul et al., 1990). Automated structure prediction was attempted using Robetta
703 (Song et al., 2013), RaptorX (Kallberg et al., 2012), Swiss-Model (Waterhouse et al., 2018),
704 PhyRe2 (Kelley et al., 2015), I-Tasser (Zhang, 2009), PsiPred (Buchan et al., 2013) and PS(2)
705 (Huang et al., 2015) servers. Four templates were selected for MgACSB modeling: 1ULT, (a
706 long chain fatty acyl-CoA synthetase homodimer from *Thermus thermophiles*) (Hisanaga et
707 al., 2004); 1PG4 (acetyl-CoA synthetase from *Salmonella enterica*) (Gulick et al., 2003);
708 5MSC (the A domain of carboxylic acid reductase from *Nocardia iowensis*) (Gahloth et al.,
709 2017); 3EQ6, a human acyl-CoA synthetase medium-chain family member (Kochan et al.,
710 2009). The sequences of these four template proteins were aligned with MgACSBG in
711 Supplemental Fig. S4. 3D structural homology models were built with Modeller (Sali and
712 Blundell, 1993) for the full length MgACSBG protein, from residue M1 to A649. Modeller
713 being able to use multiple structure alignments, four models were built using different
714 association of template proteins: M1pg4 from 1PG4 alone, M3eq6 from 3EQ6 alone and two
715 consensus models Mcons1 from 1PG4, 1ULT and 5MSC and Mcons2 from all four protein

716 templates (Supplemental data 1 to 4). Positioning of CoA and AMP in the models was based
717 on their neighboring residues in structures 1PG4 and 3EQ6 (Supplemental Tables S2A and
718 S2B) using a transformation matrix to orient these residues in the X-ray structure of 1PG4
719 towards their homologs in the models of MgACSBG. In brief, to position CoA, backbone
720 atoms of residues 305, 356, 357, 360, etc. in 1PG4 were translated and rotated to minimize
721 the root-mean-square (rms) deviation with residues 259, 310, 311, 314, etc. in the model of
722 MgACSBG. The same transformation was applied on the CoA molecule present in 1PG4 to
723 properly insert it in the models. An AMP molecule, also present in 1PG4, was positioned in
724 the two models M1pg4 and Mcons1, in the same way. Since 3EQ6 also contains both CoA
725 and AMP, the positioning of CoA and AMP in models M3eq6 and Mcons2 was done using
726 the matrix transformations pertaining to 3EQ6. As an example of a fatty acid, α -linolenic acid
727 (18:3; C₁₈H₃₀O₂ *cis*- Δ 9,12,15) was used. The lipid was initially placed manually inside the
728 structures with the head in the direction of AMP, respecting its proximity with CoA and
729 profiting of hydrophobic holes seen in the structures. The final models were finally energy
730 minimized with the molecular dynamics program CHARMM (Brooks et al., 2009) using
731 distance restraints between residues listed in Supplemental Table S2A and S2B and AMP or
732 CoA. All missing parameters in the CHARMM force field were obtained from the
733 SwissParam server (Zoete et al., 2011). All models were checked for allowed residues in the
734 Ramachandran plot (Ramachandran et al., 1963) and corrected in case of presence of *D*-amino
735 acids or *cis*-peptide bonds with Procheck (Laskowski et al., 1993). Finally, all models were
736 subject to 1 ns Langevin molecular dynamic (MD) simulation at 300 K to regularize the
737 structures before a final minimization. A summary of model energies and number of
738 misplaced residues is shown in Supplemental Table S3. Three models M1pg4, M3eq6 and
739 Mcons1, have similar final energy and correctly respect the restraints. Mcons2 shows a larger
740 energy due mainly to the more difficult respect of distance restraints. All of them present in

741 regions distant from the active site, long unstructured loops and some amino acids in
742 disallowed regions of the Ramachandran plot (Ramachandran et al., 1963). All models are
743 provided as Supplemental data (model) 1 to 4.

744 ***Yeast expression of MgACSBG variants***

745 Strains and growth media.

746 The isogenic *Saccharomyces cerevisiae* strains WT (YB332: MATa ura3 leu2 his3A200 ade2
747 lys2-801) and *faa1Δfaa4Δ* (YB525: MATa ura3 leu2 his3Δ200 ade2 lys2-801 *faa1Δ::HIS3*;
748 *faa4Δ::LYS2*) used in this study were kindly provided by Dr. Paul Black (University of
749 Nebraska at Lincoln, USA) and have been described earlier (Johnson et al., 1994; Johnson et
750 al., 1994). After transformation, all the strains described in the present work were grown on
751 synthetic media containing 6.7 g.L⁻¹ of Yeast Nitrogen Base (YNB, MP biomedical), 0.77
752 g.L⁻¹ of CSM-URA (MP biomedical) and g.L⁻¹ of either raffinose or galactose, as described.
753 In complementation experiments, the media were supplemented with 22.5 μM cerulenin
754 (Sigma-Aldrich), 100 μM free fatty acid (FFA) and 0.2% (v/v) final concentration TWEEN
755 80 (Sigma-Aldrich). The different FFAs were dissolved in 100% TWEEN 80 at a
756 concentration of 50 mM. Cerulenin was dissolved in acetone at 45 mM. As controls, media
757 without FFAs and/or cerulenin were also supplemented with 0.2% (v/v) TWEEN 80 and/or
758 acetone.

759 Cloning of the coding sequences of variants of *MgACSBG*.

760 The three variants of *MgACSBG* (*MgACSBG*, *MgACSBG#5* and *MgACSBG#31*) were
761 cloned into the PstI-BamHI cloning site of the YEplac195 backbone (X75459.1) under the
762 control of a galactose inducible promoter (pGAL1) and with the ADH1 terminator (t-ADH1).
763 A 6× His tag was cloned in frame with the genes of interest in 3', upstream and in frame with
764 the stop codon. Cloning was performed using the NEB Gibson Assembly Master Mix
765 following manufacturer's instructions. Fragments of the pGAL1 promoter, the t-ADH1

766 terminator, and WT or mutated MgACSBG coding sequences were amplified by polymerase
767 chain reaction (PCR) performed with Thermo Scientific Phusion High-Fidelity DNA
768 Polymerase, following the manufacturer's instructions. The assembled plasmids were
769 transformed in MAX Efficiency DH5 α Competent Cells. Ampicillin resistant *Escherichia coli*
770 colonies were PCR screened using pGAL1 and t-ADH1 flanking primers to validate the insert
771 size. Two PCR positive colonies per construct were sequenced. The sequence-validated
772 plasmids were transformed in the *faa1 Δ faa4 Δ* *Saccharomyces cerevisiae* double mutant by
773 the Lithium acetate method (Gietz and Schiestl, 2007) and selected on CSM-Ura. As controls,
774 WT and *faa1 Δ faa4 Δ* strains were transformed with the empty YEplac195 vector.

775 Three clones per transformation were used for complementation tests. For each experiment,
776 one colony was inoculated in 2.7 mL of CSM-URA + raffinose and grown over-night at 30°C
777 at 250 rpm. Transgene expression was induced by the addition of galactose 2% (p/v) final
778 concentration and incubation for 6 hours before measurement of the optical density at 600 nm.
779 Time course was performed in 96-well microplates (Thermo Scientific Nunc MicroWell 96-
780 Well, Nunclon Delta-Treated, Flat-Bottom Microplate) filled with 200 μ l of culture in
781 galactose medium supplemented or not by FA or cerulenin and set at an OD₆₀₀ = 0.3. The
782 microplate was incubated at 30 \pm 0.5°C in an Infinite M1000 PRO microplate reader (Tecan)
783 set for plate orbital shake for 15 seconds every 20 minutes and absorbance measurements
784 (OD₆₀₀) every 60 minutes, with four measurements per well. To the OD₆₀₀ reads from
785 experimental wells, the OD₆₀₀ measured in blank wells containing the culture media only
786 were subtracted to obtain adjusted OD₆₀₀ values. Such values were transformed using an
787 equation experimentally calculated from a calibration curve using an Eppendorf
788 BioPhotometer UV/Vis Spectrophotometer.

789 Protein expression levels were evaluated by immunoblot analyses. After induction, an amount
790 of cells corresponding to an OD₆₀₀ of 50 was retrieved by a centrifugation of 3 minutes at 600

791 ×g and washed with one milliliter of sterile water. Cells were resuspended in 200 µL of
792 protein extraction buffer (150 mM Tris-HCl pH6.8, 8M urea, 1 mM EDTA, 1 mM DTT), and
793 100 µL of acid-washed 0.5 µm glass beads were added. Cells were heated for 5 minutes at
794 70°C and ground using the PreCellys Evolution (Bertin Instruments) by 3 cycles of 20 s of
795 agitation at 10000 r.p.m. with pauses of 30 s between each cycle. The heating and grinding
796 steps were performed twice and samples were centrifuged 5 minutes at 5000 ×g at 4°C. The
797 supernatant was retrieved, and proteins were quantified using Bradford Assay (BioRad)
798 according to manufacturer's instructions. Twenty micrograms of total proteins were used for
799 immunoblot analysis. Proteins were separated on Bolt Bis-Tris Plus 4-12% (Life
800 technologies) polyacrylamide gels in MES SDS Novex Bolt migration buffer (Life
801 technologies) and then transferred in liquid in Novex Bolt Transfer buffer (Life technologies)
802 according to manufacturer's instructions. Immunoblots were performed with anti-His C-Term
803 antibodies (Invitrogen) to detect MgACSBG expression and anti-Kar2 antibodies (Santa Cruz
804 Biotechnology) for loading control. Revelation was performed with Clarity Western Blot ECL
805 substrate (BioRad) using an ImageQuant800 imager (Amersham).

806 **Accession numbers**

807 Naga_100014g59; Naga_100012g66; Naga_101051g1; Naga_100047g8; Naga_100649g1;
808 Naga_100035g43; Phatr3_J20143; Phatr3_J12420; Phatr3_J54151; Phatr3_J45510;
809 Phatr3_J17720; NP_524698; NP_001285923; NP_444408; XP_015155301; Q96GR2;
810 Q5FVE4; F1NLD6.

811 **Supplemental Data**

812 **Supplemental Figure S1.** Overview of glycerolipid biosynthesis.

813 **Supplemental Fig. S2.** Multiple alignment of ACSBG proteins sequences from animals and
814 heterokonts.

815 **Supplemental Fig. S3.** Secondary structure prediction of MgACSBG (Naga_100014g59).

816 **Supplemental Fig. S4.** Sequence Alignment of MgACSBG (Naga_100014g59 or NAGA)
817 with proteins of known 3D-structure.

818 **Supplemental Fig. S5.** Expression of MgACSBG, MgACSBG#5 and MgACSBG#31 in the
819 *S. cerevisiae faa1Δfaa4Δ* double mutant.

820 **Supplemental Table S1.** In depth analysis of phosphatidylcholine (PC) and betaine lipid
821 (DGTS) fatty acid profiling by mass spectrometry.

822 **Supplemental Table S2.** Aminoacids in MgACSBG sequence predicted to be in the vicinity
823 of Coenzyme A (CoA) and Adenosine monophosphate (AMP).

824 **Supplemental Table S3.** Evaluation of 3D-models of MgACSBG.

825 **Supplemental Model 1.** Model (1) acs4_3eq6_ac_3eq6_lipr_d1_min, in pdb format

826 **Supplemental Model 2.** Model (2) acs4_al2_ac_3eq6_lipr_d1_min, in pdb format

827 **Supplemental Model 3.** Model (3) acs4_all_ac_1pg4_lipr_min, in pdb format

828 **Supplemental Model 4.** Model (4) acs4_1pg4_ac_1pg4_lipr_min, in pdb format

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838 coauthors, Serge Crouzy, who unfortunately passed away in April 2020.

839 **Figure Legends**

840 **Figure 1: Central role of acyl-CoAs in cell metabolism.** (A) In this simplified scheme, acyl-
841 CoAs are initially generated from fatty acids synthesized *de novo* by the action of specific
842 ACSs. (B) Acyl-CoAs can be esterified to the *sn*-1 and *sn*-2 position of glycerol-3-phosphate,
843 thus generating phosphatidic acid, which serves as a substrate for diacylglycerol and other
844 membrane glycerolipids. Some specific acyl-CoAs, such as 18:3-CoA, can be further
845 elongated and transferred to specific glycerolipids for additional desaturations (dashed lines),
846 thus producing very-long chain polyunsaturated fatty acids (VLC-PUFAs). (C) Acyl-CoAs
847 can be esterified to the *sn*-3 position of diacylglycerol to form triacylglycerol. (D)
848 Furthermore, acyl-CoAs can be used for the synthesis of a variety of other acyl-molecules,
849 such as sphingolipids, acylated proteins, sterol esters, and signaling molecules. (E) In this
850 scheme, fatty acids hydrolyzed from membrane glycerolipids or triacylglycerol can re-enter
851 the pool of acyl-CoAs by the action of other specific ACSs. (F) Eventually, Acyl-CoAs can
852 be degraded via the beta-oxidation pathway occurring in mitochondria and/or peroxisomes.
853 Adapted from (Coleman et al., 2002).

854 **Figure 2. Motif conservation in Acyl-CoA synthetases of the Bubblegum family**
855 **(ACSBGs) defined by Steinberg et al., 2000.** Residues conserved in more than 50% of the
856 sequences are shown in black boxes. This motif is based on a comprehensive comparison of
857 ACS sequences in eukaryotes (Steinberg et al., 2000). Alignment includes sequences from
858 fruit fly (*Drosophila melanogaster*; dmBG AAF44848 and dmBG-H1 AAF44850), mouse
859 (*Mus musculus*; mmBG AA111606), human (*Homo sapiens*; HsACBG1 and HsACBG2),
860 chicken (*Gallus gallus*, GgACSBG1 and GgACSBG2), *Microchloropsis gaditana*
861 (MgACSBG or Naga_100014g59) and *Phaedoctylum tricornutum* (Phatr3_J45510).

862 **Figure 3: Phylogenetic comparison of acyl-CoA synthetases (ACS) from *M. gaditana* and**
863 ***P. tricornutum*, with ACSBG sequences from Metazoa.** All genes annotated as acyl-CoA
864 synthetases or ligases from *M. gaditana* (Naga_100014g59, MgACSBG/Naga_100014g59;
865 Naga_100012g66; Naga_101051g1; Naga_100047g8; Naga_100649g1 and
866 Naga_100035g43) and *P. tricornutum* (Phatr3_J20143, ACS1; Phatr3_J12420, ACS2;
867 Phatr3_J54151, ACS3; Phatr3_J45510, PtACSBG or ACS4; and Phatr3_J17720, ACL1) were
868 compared with ACSBG sequences from *D. melanogaster* (DmACSBGa, NP_524698;
869 DmACSBGc, NP_001285923); *H. sapiens* (HsACBG1, Q96GR; HsACBG2, Q5FVE4); *M.*
870 *musculus* (MmACSBG1, NP_444408); *G. gallus* (GgACSBG1, F1NLD6; GgACSBG2,
871 XP_015155301). The tree was calculated using a Bayesian inference method (MrBayes) as
872 described in the Methods section, a Poisson model for amino acid substitutions, four Markov
873 chain Monte Carlo (MCMC) chains run for 10,000 generations, sampling every 10
874 generations and a 50% majority rule consensus tree.

875 **Figure 4: MgACSBG mutants.** (A) Mutant selection. Following *M. gaditana* co-
876 transformation with the two specifically designed TALE-N subunits, obtained clones were
877 analyzed by treatment with a T7 endonuclease to detect cleaved DNA at mismatched
878 positions and assess occurrence of genome editing at the target locus. When this test allowed
879 detecting a mutation, the cell population was sub-cloned onto a new selective plate, and
880 colonies arising from single cells were sequenced at the MgACSBG inside the FokI activity
881 region (frame), resulting in the appearance of novel codons. Three mutant lines harboring
882 mutated endogenous *MgACSBG* are shown, called here MgACSBG#5, MgACSBG#31 and
883 MgACSBG#40. Only two to three aminoacids were modified in the endogenous proteins. (B)
884 Point mutations in MgACSBG#5, MgACSBG#31 and MgACSBG#40. In grey boxes, wild
885 type (WT) residues at the TALE-N nucleotidic target; in black boxes, inserted mutations.

886 **Figure 5:** Compared analysis of MgACSBG#5 and MgACSBG#31 with untransformed wild-
887 type cells (WT) and cells transformed with an empty vector (EV). **(A)** Growth was measured
888 by cell counting as described in the Methods section at days 3, 7 and 12 following inoculation
889 (D3, D7 and D12, respectively). D3 corresponds to nutrient replete cultivation, whereas D7
890 and D12 correspond to nitrogen-starved condition. **(B)** Fatty acid content per million cells at
891 D3, D7 and D12 following inoculation. **(C)** Triacylglycerol content, in mol% of total
892 glycerolipids, at D3, D7 and D12 following inoculation. **(D)** Fatty acid profile at D3. Total
893 FAs were extracted and, following methanolysis, obtained FA methyl esters were analyzed
894 and quantified by gas chromatography coupled with ion flame detection (GC-FID), as
895 described in the Methods section. **(E)** Glycerolipid profile at D3. Each glycerolipid class was
896 analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MSMS) and
897 quantified as described in the Methods section. DAG, diacylglycerol; DGDG,
898 digalactosyldiacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; FA, fatty
899 acid; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE,
900 phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG,
901 sulfoquinovosyldiacylglycerol; TAG, triacylglycerol. Data are the average of 4 replicates.
902 Error bars, standard deviation. (*), *P*-value < 0.05; student's t-test using WT as a reference.

903 **Figure 6: Comparison of the acyl profiles of glycerolipid classes in MgACSBG#5,**
904 **MgACSBG#31, untransformed wild-type cells (WT) and cells transformed with an**
905 **empty vector (EV).** (A) Phosphatidylcholine, PC. (B) Diacylglyceryl-N,N,N-
906 trimethylhomoserine, DGTS. (C) Phosphatidylethanolamine, PE. (D) Phosphatidylglycerol,
907 PG. (E) Sulfoquinovosyldiacylglycerol, SQDG. (F) Monogalactosyldiacylglycerol, MGDG.
908 (G) Digalactosyldiacylglycerol, DGDG. (H) Triacylglycerol, TAG. Major molecular species
909 corresponding to combined mass given in number of carbons and of double bonds are
910 indicated, based on previous regioselective analysis of *M. gaditana* glycerolipids (Alboresi et
911 al., 2016). Molecular species of which the proportion increases in the membrane lipids of
912 MgACSBG#5 and MgACSBG#31 are shown in red, whereas those of which the proportion
913 decreases are shown in green. Data are the average of 4 replicates and are given in mol%.
914 Error bars, standard deviation. (*), *P*-value < 0.05; student's t-test using WT as a reference.

915 **Figure 7: Model for ACSBG role in *M. gaditana*.** The scheme shows the major fatty acids
916 in major glycerolipid classes, i.e. PC, DGTS, PE and TAG in the ER, and MGDG in the
917 plastid. Each membrane glycerolipid class is shown as a grey block. TAG is shown in a grey
918 circle. **(A)** *De novo* synthesis of fatty acids (FAs) in the stroma of the plastid. The action of
919 FA synthetase of type II (FAS II) leads to the production of 16:0-ACP, which can be
920 desaturated by a palmitoyl ACP desaturase (PAD) into 16:1-ACP. These acyls can be
921 exported to the cytosol, possibly as free fatty acids, through the four limiting membranes of
922 the plastid. **(B)** ACSBG phenotype is consistent with a role in the production of 16:1-CoA.
923 Unknown ACS isoforms (ACSx) catalyze the production of 16:0-CoA. **(C)** The 16:1 is
924 incorporated at the sn-2 position of ER lipids via LPAT1 in *N. oceanica* (Nobusawa et al.,
925 2017). Impairment of ACSBG alters this process. **(D)** The 16:0-CoA is elongated into 18:0-
926 CoA by the action of D0-ELO isoforms, as a committing step in the 16:0-to-20:5 synthesis
927 pathway (Dolch et al., 2017). **(E)** VLC-PUFA synthesis, starting with the 18:0-to-18:3
928 desaturation on PC by the stepwise action of an ERD9FAD, an ERD12FAD and an
929 ERD6FAD. Since PC accumulates 18 precursors (18:1 and 18:2), we consider that the
930 mutants are likely impaired downstream, at the level of 18:3 elongation, leading therefore to
931 the positioning of ACSBG at the level of 18:3-CoA production, feeding the Δ 6-ELO isoform
932 producing 20:3-CoA. Downstream processes, i.e. 20:3-to-20:5 desaturations occurring on
933 DGTS would be consistently impaired, with a decrease in the end-product, 20:5. **(F)** Some
934 20:3-to-20:5 desaturation processes occurring in DGTS operate also in PE. The slowing down

935 of 20:5 synthesis also occurs in PE. **(G)** As a result, following the mass action law, the
936 slowing down occurring in the 16:0-to-20:5 pathway is expected to lead to an over-
937 accumulation of 16:0-CoA. Excess 16:0-CoA can be stored as TAG, via the DGAT/Kennedy
938 pathway. **(H)** An important assumption for this model is that a positive feedback is exerted on
939 the plastid, exporting most its FAs, and explaining that the omega pathway is activated to
940 provide sufficient 20:5 to plastid glycerolipids to compensate the lack of 16-carbon substrates.
941 AAS, acyl-ACP synthetase; ACS, acyl-CoA synthetase; DGAT, diacylglycerol
942 acyltransferase; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; FAD, fatty acid
943 desaturase; MGDG, monogalactosyldiacylglycerol; PAD, palmitoyl ACP desaturase; PC,
944 phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

945 **Figure 8: Comparison of the acyl-CoA profiles in MgACSBG#5, MgACSBG#31,**
946 **untransformed wild-type cells (WT) and cells transformed with an empty vector (EV).**
947 Cells were cultivated in nutrient replete conditions as described in the Methods section (D3).
948 Mass-spectrometry based analysis and data processing were performed by Rothamsted
949 Research, UK. Data are the average of 2 replicates. Error bars, standard deviation. (*), P-
950 value < 0.05; student's t-test using WT as a reference.

951 **Figure 9. 3D model of MgACSBG bound to CoA, AMP and α -linolenic acid (18:3).** Four
952 models of MgACSBG were obtained based on similarity with acetyl- and acyl-CoA
953 synthetase of known 3D structure, as described in the Methods section. The Mcons1 model of
954 MgACSBG is shown with the visualization program VMD (Humphrey et al., 1996). The
955 backbone of residues around AMP and CoA, listed in Supplemental Table S2A and S2B, are
956 shown in licorice. The IGF triad is represented in pink (I96, G97, F98). The conserved Y216-
957 K225 active site is shown in licorice mode. The position of IGF close to 18:3 is similar in all
958 four MgACSBG models. Abbreviations: COA, Coenzyme A; AMP, adenosine
959 monophosphate; LIP, α -linolenic acid.

960 **Figure 10. Growth complementation of a yeast mutant defective in acyl-CoA activity by**
961 **MgACSBG variants. (A)** WT yeast strain growth curves in galactose medium (Cer-, green
962 circles, positive control), with the addition of 22.5 μ M cerulenin only (Cer+, grey circles,
963 negative control), or with the addition of 22.5 μ M cerulenin and 100 μ M of the following free
964 fatty acids, 14:0, 16:0, 18:0, 18:1, 18:2 and 18:3 (curves ranging from blue to red). **(B)**
965 Growth curves of MgACSBG complemented *faa1 Δ faa4 Δ* double yeast mutant in galactose
966 medium supplemented by 22.5 μ M cerulenin and 100 μ M of FA (curves ranging from blue to
967 red). Medium with 22.5 μ M cerulenin but FA was used as a negative control (Cer+, grey
968 circles). Average (+/- standard deviation) of biological (n = 3, independent clones) as well as
969 technical (n = 3) replicates are presented. **(C)** and **(D)** Growth curves of the *faa1 Δ faa4 Δ*
970 double mutant complemented with MgACSBG (green squares), MgACSBG#5 (blue squares),
971 and MgACSBG#31 (purple squares) in presence of 22.5 μ M cerulenin and 100 μ M 14:0 (C) or
972 16:0 (D). The *faa1 Δ faa4 Δ* double mutant without complementation was used as negative
973 control and represented by grey circles. Average (+/- standard deviation) of biological (n = 3,
974 independent clones) replicates are presented.

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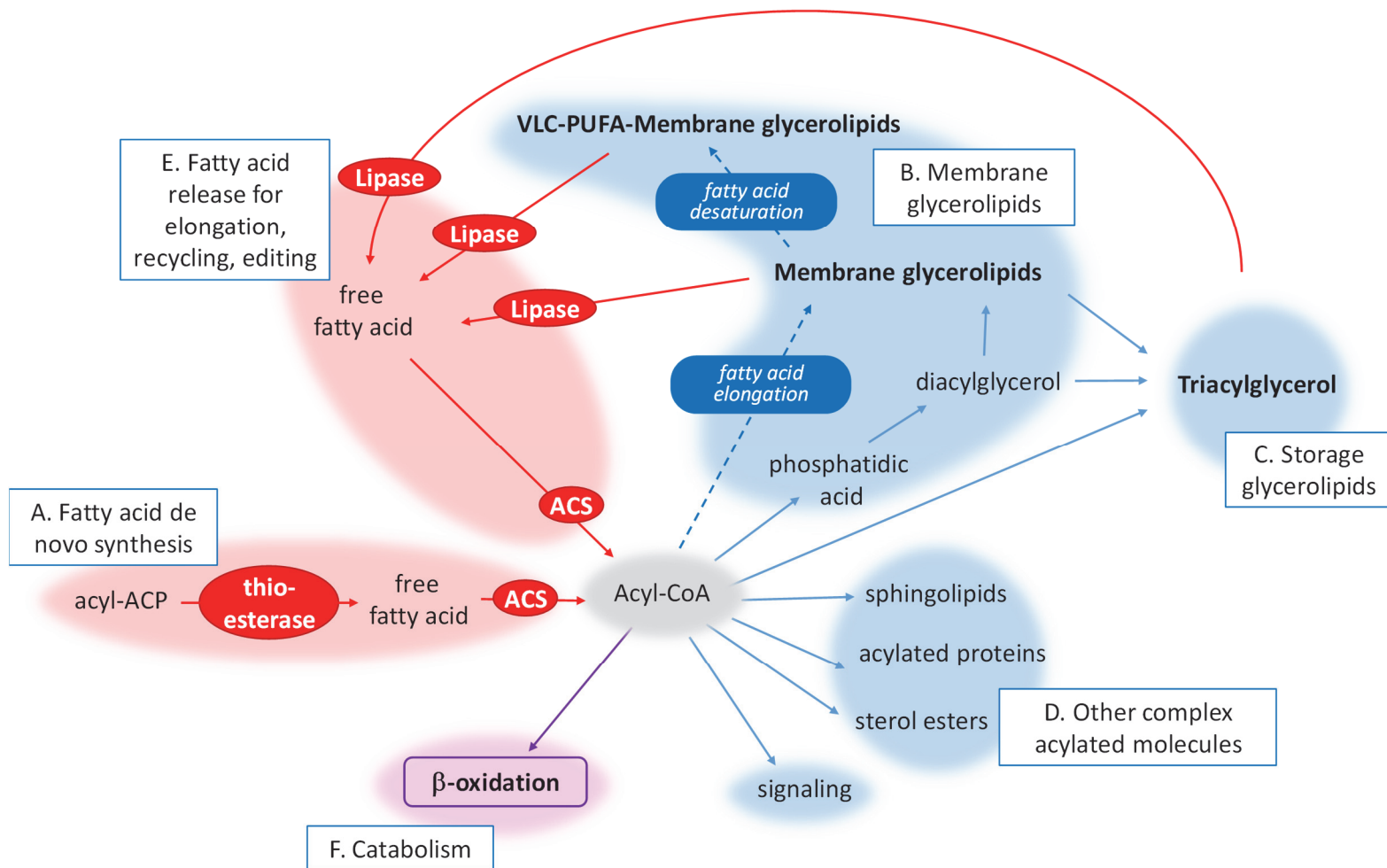


Figure 1: Central role of acyl-CoAs in cell metabolism. (A) In this simplified scheme, acyl-CoAs are initially generated from fatty acids synthesized *de novo*, by the action of specific ACSs. (B) Acyl-CoAs can be esterified to the *sn*-1 and *sn*-2 position of glycerol-3-phosphate, thus generating phosphatidic acid, which serves as a substrate for diacylglycerol and other membrane glycerolipids. Some specific acyl-CoAs, such as 18:3-CoA can be further elongated and transferred to specific glycerolipids for additional desaturations (dashed lines), thus producing very-long chain polyunsaturated fatty acids (VLC-PUFAs). (C) Acyl-CoAs can be esterified to the *sn*-3 position of diacylglycerol to form triacylglycerol. (D) Furthermore, acyl-CoAs can be used for the synthesis of a variety of other acyl-molecules, such as sphingolipids, acylated proteins, sterol esters, signaling molecules. (E) In this scheme, fatty acids hydrolyzed from membrane glycerolipids or triacylglycerol can re-enter the pool of acyl-CoAs by the action of other specific ACSs. (F) Eventually, Acyl-CoAs can be degraded via the beta-oxidation pathway occurring in mitochondria and/or peroxisomes. Adapted from (Coleman et al., 2002).

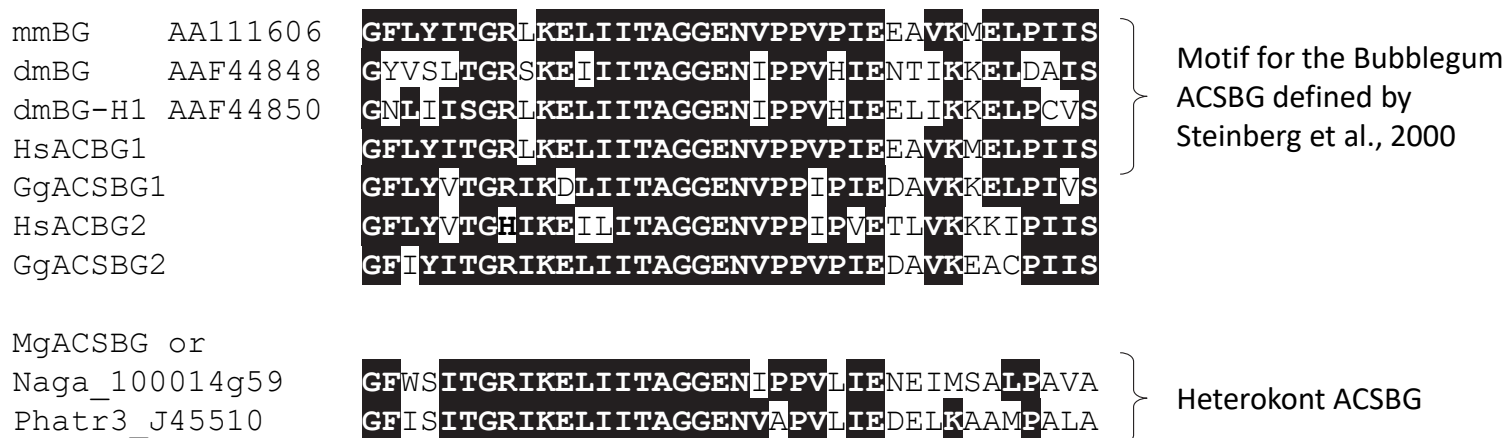


Figure 2. Motif conservation in Acyl-CoA synthetases of the Buble gum family (ACSBGs) defined by Steinberg et al., 2000. Residues conserved in more than 50% of the sequences are shown in black boxes. This motif is based on a comprehensive comparison of ACS sequences in eukaryotes (Steinberg et al., 2000).

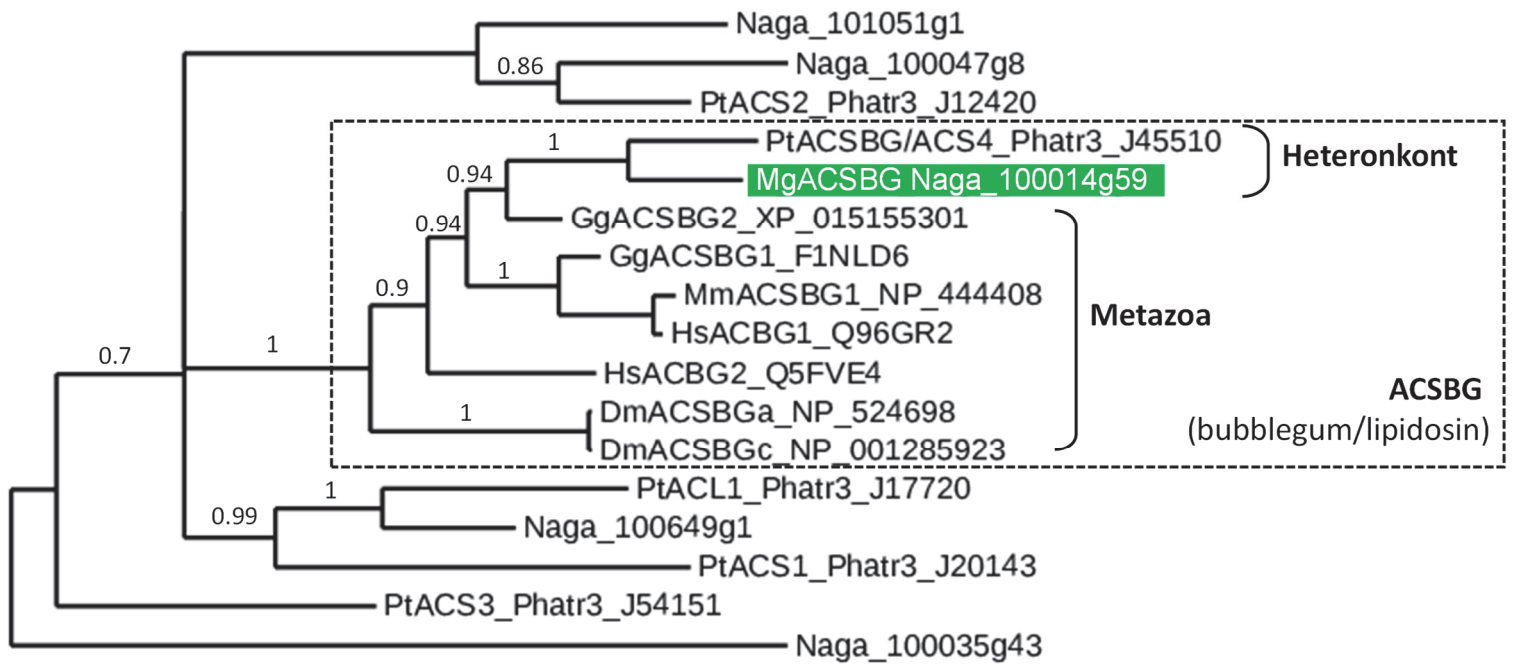


Figure 3: Phylogenetic comparison of acyl-CoA synthetases (ACS) from *M. gaditana* and *P. tricornutum*, with ACSBG sequences from Metazoa. All genes annotated as acyl-CoA synthetases or ligases from *M. gaditana* (Naga_100014g59, MgACSBG/Naga_100014g59; Naga_100012g66; Naga_101051g1; Naga_100047g8; Naga_100649g1 and Naga_100035g43) and *P. tricornutum* (Phatr3_J20143, ACS1; Phatr3_J12420, ACS2; Phatr3_J54151, ACS3; Phatr3_J45510, PtACSBG or ACS4; and Phatr3_J17720, ACL1) were compared with ACSBG sequences from *D. melanogaster* (DmACSBGa, NP_524698; DmACSBGc, NP_001285923); *H. sapiens* (HsACBG1, Q96GR; HsACBG2, Q5FVE4); *M. musculus* (MmACSBG1, NP_444408); *G. gallus* (GgACSBG1, F1NLD6; GgACSBG2, XP_015155301). The tree was calculated using a Bayesian inference method (MrBayes) as described in the Method section, a Poisson model for amino acid substitutions, four Markov chain Monte Carlo (MCMC) chains run for 10,000 generations, sampling every 10 generations and a 50% majority rule consensus tree.

A

```

MgACSBG      GGATTTCCTGCATGGTGCCATTAATTTA---ATCGGTTTCAACTCCCGGAATGGCTATTGCCAACTGTG
MgACSBG#5    GGATTTCCTGCATGGTGCCATTAATTTA---TTTCGGTTAAACTCCCGGAATGGCTATTGCCAACTGTG
MgACSBG#31   GGATTTCCTGCATGGTGCCATTAATTTA---CGGGTTTTTAACCTCCCGGAATGGCTATTGCCAACTGTG
MgACSBG#40   GGATTTCCTGCATGGTGCCATTAATTTA---CGGGTTTTTTCAA---AACTCCCGGAATGGCTATTGCCAACTGTG

```

Left TAL binding site
Fok1 activity region
Right TAL binding site

B

```

1                               80
MgACSBG      MPKYTTTVASGEVDLRIEKEGPGSWAPKTVFQVFEETVKKYGDSPALHYKKVPHGGSLATTEWSSYTWREYYDLTLEFCK
MgACSBG#5    MPKYTTTVASGEVDLRIEKEGPGSWAPKTVFQVFEETVKKYGDSPALHYKKVPHGGSLATTEWSSYTWREYYDLTLEFCK
MgACSBG#31   MPKYTTTVASGEVDLRIEKEGPGSWAPKTVFQVFEETVKKYGDSPALHYKKVPHGGSLATTEWSSYTWREYYDLTLEFCK
MgACSBG#40   MPKYTTTVASGEVDLRIEKEGPGSWAPKTVFQVFEETVKKYGDSPALHYKKVPHGGSLATTEWSSYTWREYYDLTLEFCK

81=====***                               160
MgACSBG      SLLSLGFPAHGAINLIGF--NSPEWLIANCGAIAAGGVGVGIYTSNGVDACKYITEHSEAEVVVVENAKQLEKYLKIAKEL
MgACSBG#5    SLLSLGFPAHGAINLRFI--NSPEWLIANCGAIAAGGVGVGIYTSNGVDACKYITEHSEAEVVVVENAKQLEKYLKIAKEL
MgACSBG#31   SLLSLGFPAHGAINLRFV--NSPEWLIANCGAIAAGGVGVGIYTSNGVDACKYITEHSEAEVVVVENAKQLEKYLKIAKEL
MgACSBG#40   SLLSLGFPAHGAINLRFQ--NSPEWLIANCGAIAAGGVGVGIYTSNGVDACKYITEHSEAEVVVVENAKQLEKYLKIAKEL

                               motif I (AMP-binding)
                               <----->
                               240
MgACSBG      PRLKALVIYSGTAEGYKCDVPIYSWKDFMALGSGVKDEAVRARIEAQRPGHCCTLIYTSGTTGPPKAVMISHDNLWTWVK
MgACSBG#5    PRLKALVIYSGTAEGYKCDVPIYSWKDFMALGSGVKDEAVRARIEAQRPGHCCTLIYTSGTTGPPKAVMISHDNLWTWVK
MgACSBG#31   PRLKALVIYSGTAEGYKCDVPIYSWKDFMALGSGVKDEAVRARIEAQRPGHCCTLIYTSGTTGPPKAVMISHDNLWTWVK
MgACSBG#40   PRLKALVIYSGTAEGYKCDVPIYSWKDFMALGSGVKDEAVRARIEAQRPGHCCTLIYTSGTTGPPKAVMISHDNLWTWVK

                               motif IV (FA gate domain)
                               <***->                               * * * * 320
MgACSBG      NFVASLPFTLTCEDRSVSYLPLSHVAAQMLDIHCPIATGAKIYFAQPDALRGSPLVTLKDVCPITYFFGVPRVWEKIYEKM
MgACSBG#5    NFVASLPFTLTCEDRSVSYLPLSHVAAQMLDIHCPIATGAKIYFAQPDALRGSPLVTLKDVCPITYFFGVPRVWEKIYEKM
MgACSBG#31   NFVASLPFTLTCEDRSVSYLPLSHVAAQMLDIHCPIATGAKIYFAQPDALRGSPLVTLKDVCPITYFFGVPRVWEKIYEKM
MgACSBG#40   NFVASLPFTLTCEDRSVSYLPLSHVAAQMLDIHCPIATGAKIYFAQPDALRGSPLVTLKDVCPITYFFGVPRVWEKIYEKM

321                               ***                               400
MgACSBG      QEVARSTTGVKRALAQWAKAKGLEKNRRQQYGC GGGAPVGF GCAHALVLSKVKAALGLHQTKMCITSAAPIAVEILEYFA
MgACSBG#5    QEVARSTTGVKRALAQWAKAKGLEKNRRQQYGC GGGAPVGF GCAHALVLSKVKAALGLHQTKMCITSAAPIAVEILEYFA
MgACSBG#31   QEVARSTTGVKRALAQWAKAKGLEKNRRQQYGC GGGAPVGF GCAHALVLSKVKAALGLHQTKMCITSAAPIAVEILEYFA
MgACSBG#40   QEVARSTTGVKRALAQWAKAKGLEKNRRQQYGC GGGAPVGF GCAHALVLSKVKAALGLHQTKMCITSAAPIAVEILEYFA

                               motif III
                               <*****>                               480
MgACSBG      SLDIPVLELFGQSECTGPHTSNFSYAWKIGSIGRDI PGVKTQKHANMSEFCMYGRHIMMGYMKMEDKTQEAVDNEGWLHS
MgACSBG#5    SLDIPVLELFGQSECTGPHTSNFSYAWKIGSIGRDI PGVKTQKHANMSEFCMYGRHIMMGYMKMEDKTQEAVDNEGWLHS
MgACSBG#31   SLDIPVLELFGQSECTGPHTSNFSYAWKIGSIGRDI PGVKTQKHANMSEFCMYGRHIMMGYMKMEDKTQEAVDNEGWLHS
MgACSBG#40   SLDIPVLELFGQSECTGPHTSNFSYAWKIGSIGRDI PGVKTQKHANMSEFCMYGRHIMMGYMKMEDKTQEAVDNEGWLHS

                               motif II (FA binding)
                               <-----*----->                               560
MgACSBG      GDVAQVDADGFWSITGRIKELIITAGGENIPVLIENEIMSALPAVANCMVVGDKKKFLTVLLTMKAKLDDQGNPTKELN
MgACSBG#5    GDVAQVDADGFWSITGRIKELIITAGGENIPVLIENEIMSALPAVANCMVVGDKKKFLTVLLTMKAKLDDQGNPTKELN
MgACSBG#31   GDVAQVDADGFWSITGRIKELIITAGGENIPVLIENEIMSALPAVANCMVVGDKKKFLTVLLTMKAKLDDQGNPTKELN
MgACSBG#40   GDVAQVDADGFWSITGRIKELIITAGGENIPVLIENEIMSALPAVANCMVVGDKKKFLTVLLTMKAKLDDQGNPTKELN

                               motif V
                               <-->                               640
MgACSBG      KEALDIGKEIGSNASTTEQVADPHWKYFDEGLKKNSTATSNAQFVQKWSVLPDLFSEKGGELPTLKLKRSVVAEKY
MgACSBG#5    KEALDIGKEIGSNASTTEQVADPHWKYFDEGLKKNSTATSNAQFVQKWSVLPDLFSEKGGELPTLKLKRSVVAEKY
MgACSBG#31   KEALDIGKEIGSNASTTEQVADPHWKYFDEGLKKNSTATSNAQFVQKWSVLPDLFSEKGGELPTLKLKRSVVAEKY
MgACSBG#40   KEALDIGKEIGSNASTTEQVADPHWKYFDEGLKKNSTATSNAQFVQKWSVLPDLFSEKGGELPTLKLKRSVVAEKY

641      650
MgACSBG      ADVIADMYKA
MgACSBG#5    ADVIADMYKA      *: Conserved protein domain cd05933 (ACSBG_like)
MgACSBG#31   ADVIADMYKA      =: Hydrophobic domain
MgACSBG#40   ADVIADMYKA      <->: ACSBG-specific motifs

```

Figure 4: MgACSBG mutants. (A) Mutant selection. Following *M. gaditana* co-transformation with the two specifically designed TALE-N subunits, obtained clones were analyzed by treatment with a T7 endonuclease, to detect cleaved DNA at mismatched positions and assess occurrence of genome editing at the target locus. When this test allowed detecting a mutation, the cell population was sub-cloned onto a new selective plate and colonies arising from single cells were sequenced at the MgACSBG, inside the FokI activity region (frame) resulting in the appearance of novel codons. Three mutant lines harboring mutated endogenous *MgACSBG* are shown, called here MgACSBG#5, MgACSBG#31 and MgACSBG#40. Only two to three aminoacids were modified in the endogenous proteins. **(B) Point mutations in MgACSBG#5, MgACSBG#31 and MgACSBG#40.** In grey boxes, wild type (WT) residues at the TALE-N nucleotidic target; in black boxes, inserted mutations.

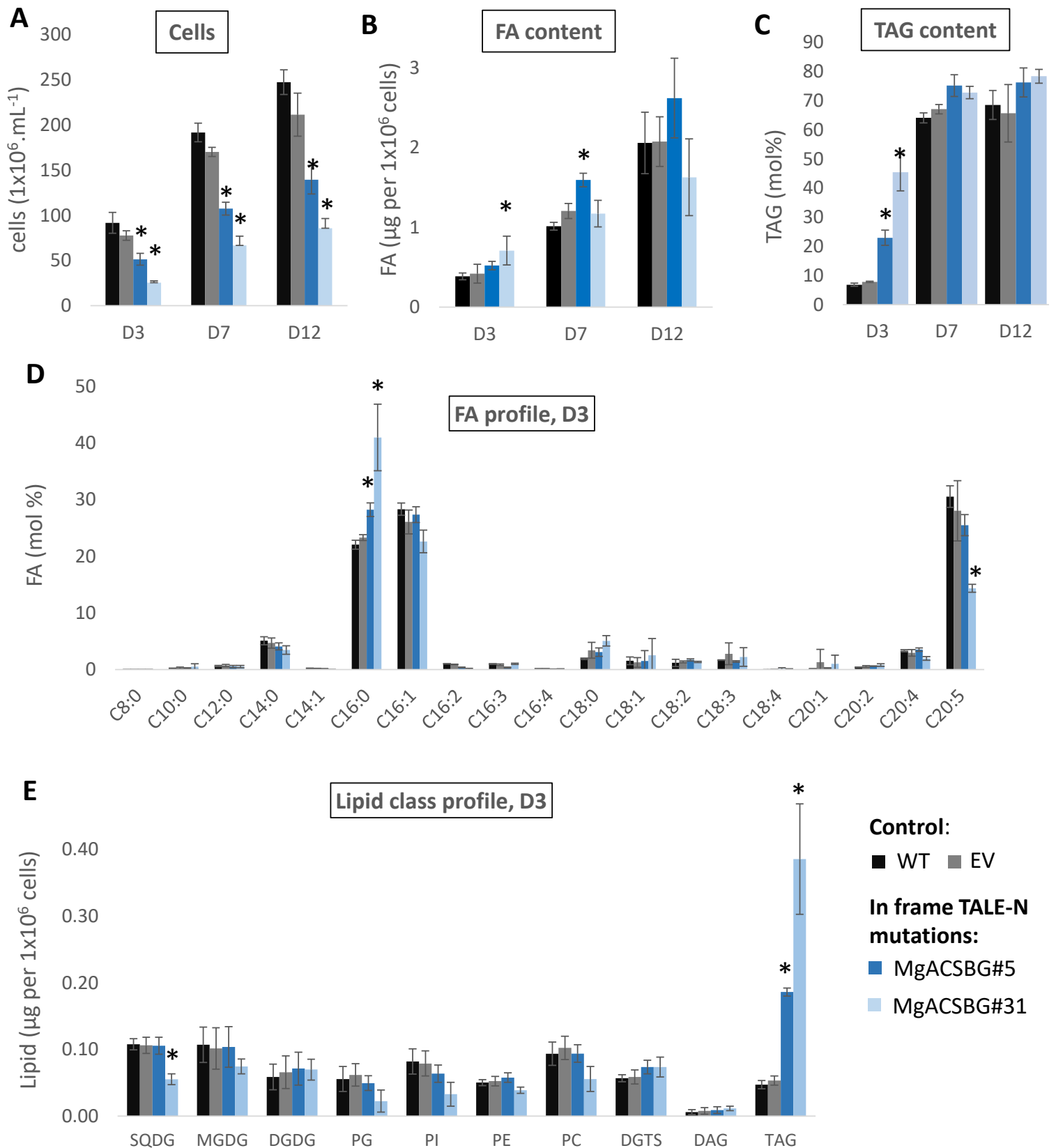


Figure 5: Compared analysis of MgACSBG#5 and MgACSBG#31 with untransformed wild type cells (WT) and cells transformed with an empty vector (EV). **(A)** Growth was measured by cell counting as described in the Method section at day 3, 7 and 12 following inoculation (D3, D7 and D12, respectively). D3 corresponds to nutrient replete cultivation, whereas D7 and D12 correspond to nitrogen-starved condition. **(B)** Fatty acid content per million cells at D3, D7 and D12 following inoculation. **(C)** Triacylglycerol content, in mol% of total glycerolipids, at D3, D7 and D12 following inoculation. **(D)** Fatty acid profile at D3. Total FAs were extracted and, following methanolysis, obtained FA methyl esters were analyzed and quantified by gas chromatography coupled with ion flame detection (GC-FID), as described in the Method section. **(E)** Glycerolipid profile at D3. Each glycerolipid class was analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MSMS) and quantified as described in the Methods section. DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGTS, diacylglycerol-N,N,N-trimethylhomoserine; FA, fatty acid; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol; TAG, triacylglycerol. Data are the average of 4 replicates. Error bars, standard deviation. (*), *P*-value < 0.05; student's t-test using WT as a reference.

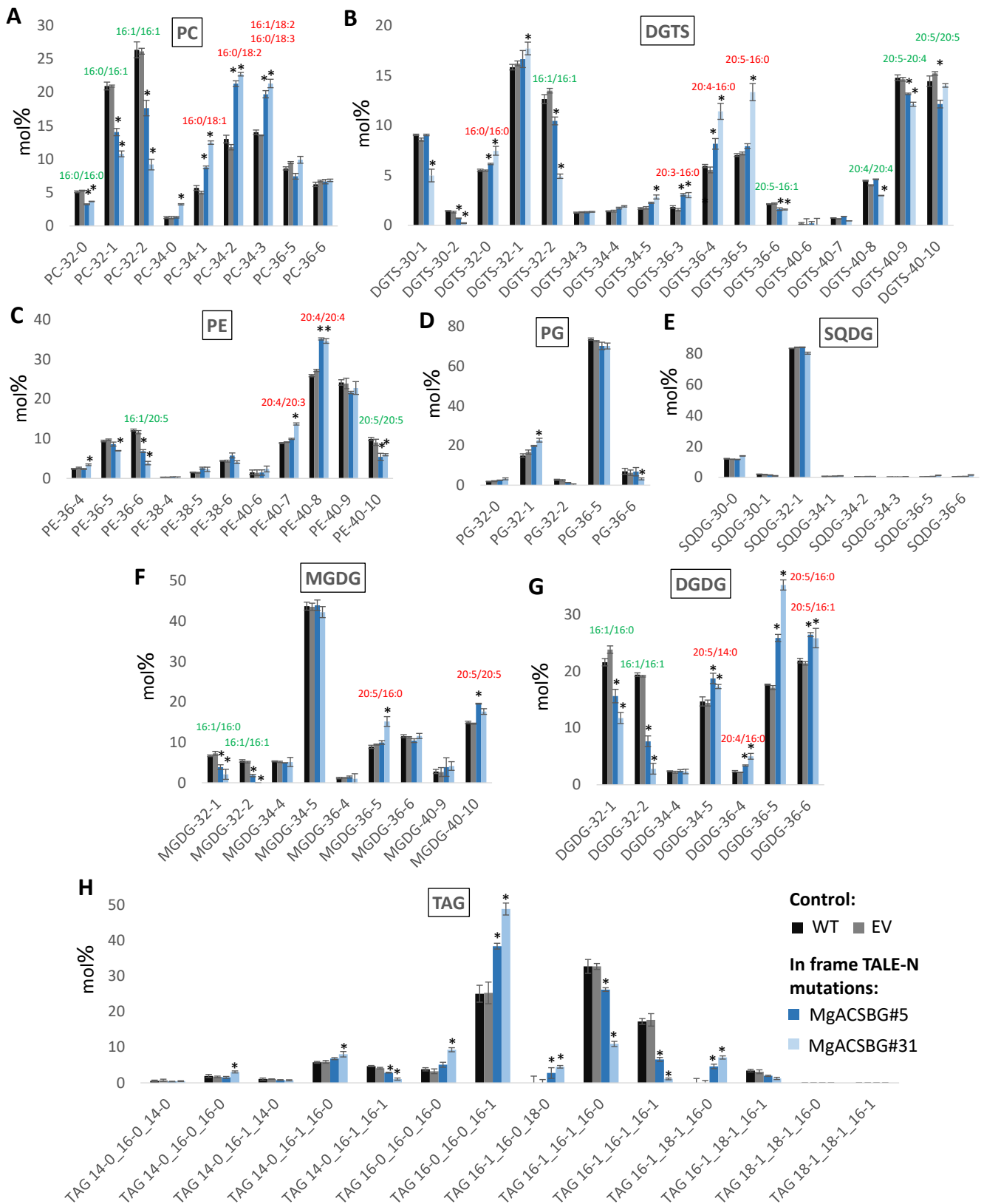


Figure 6: Comparison of the acyl profiles of glycerolipid classes in *MgACSBG#5*, *MgACSBG#31*, untransformed wild type cells (WT) and cells transformed with an empty vector (EV). (A) Phosphatidylcholine, PC. (B) Diacylglyceryl-N,N,N-trimethylhomoserine, DGTS. (C) Phosphatidylethanolamine, PE. (D) Phosphatidylglycerol, PG. (E) Sulfoquinovosyldiacylglycerol, SQDG. (F) Monogalactosyldiacylglycerol, MGDG. (G) Digalactosyldiacylglycerol, DGDG. (H) Triacylglycerol, TAG. Major molecular species corresponding to combined mass given in number of carbons and of double bonds are indicated, based on previous regioselective analysis of *M. gaditana* glycerolipids (Alboresi et al., 2016). Molecular species of which the proportion increases in the membrane lipids of *MgACSBG#5* and *MgACSBG#31* are shown in red, whereas those of which the proportion decreases are shown in green. Data are the average of 4 replicates and are given in mol%. Error bars, standard deviation. (*), P-value < 0.05; student's t-test using WT as a reference.

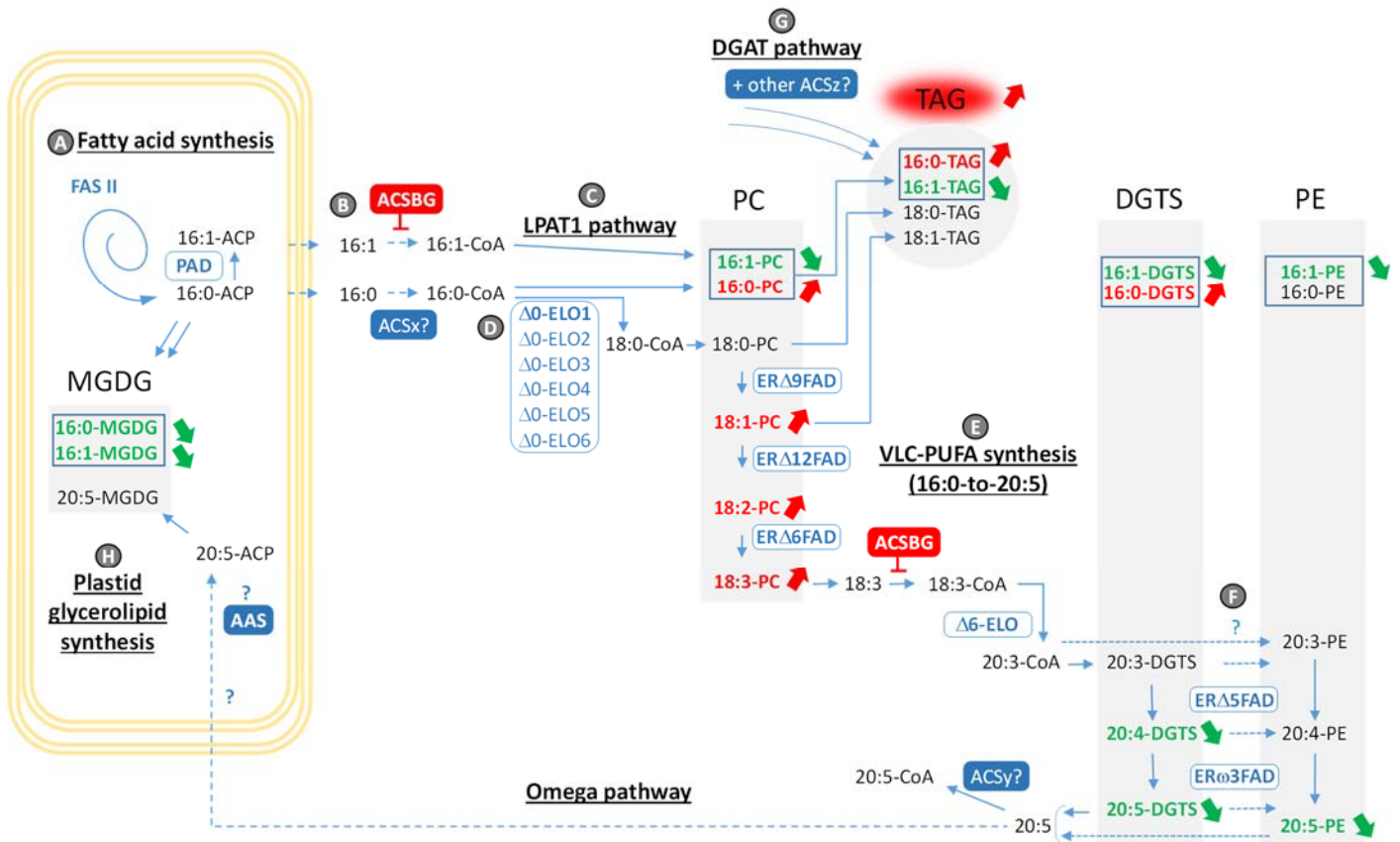


Figure 7: Model for ACSBG role in *M. gaditana*. The scheme shows the major fatty acids in major glycerolipid classes, i.e. PC, DGTS, PE and TAG in the ER, and MGDG in the plastid. Each membrane glycerolipid class is shown as a grey block. TAG is shown in a grey circle. **(A)** De novo synthesis of fatty acids (FAs) in the stroma of the plastid. The action of FA synthetase of type II (FAS II) leads to the production of 16:0-ACP, which can be desaturated by a palmitoyl ACP desaturase (PAD) into 16:1-ACP. These acyls can be exported to the cytosol, possibly as free fatty acids, through the four limiting membranes of the plastid. **(B)** ACSBG phenotype is consistent with a role in the production of 16:1-CoA. An unknown ACS isoforms (ACSx) catalyze the production of 16:0-CoA. **(C)** It was shown that 16:1 is incorporated at the sn-2 position of ER lipids via LPAT1 in *N. oceanica* (Nobusawa et al., 2017). Impairment of ACSBG alters this process. **(D)** It was shown that 16:0-CoA is elongated into 18:0-CoA by the action of D0-ELO isoforms, as a committing step in the 16:0-to-20:5 synthesis pathway (Dolch et al., 2017). **(E)** VLC-PUFA synthesis, starting by the 18:0-to-18:3 desaturation on PC, by the stepwise action of an ERD9FAD, an ERD12FAD and an ERD6FAD. Since PC accumulates 18 precursors (18:1 and 18:2) we consider that the mutants are likely impaired downstream, at the level of 18:3 elongation, leading therefore to the positioning of ACSBG at the level of 18:3-CoA production, feeding the $\Delta 6$ -ELO isoform producing 20:3-CoA. Downstream processes, i.e. 20:3-to-20:5 desaturations occurring on DGTS would be consistently impaired, with a decrease in the end-product, 20:5. **(F)** Some 20:3-to-20:5 desaturation processes occurring in DGTS operate also in PE. The slowing down of 20:5 synthesis also occurs in PE. **(G)** As a result, following the mass action law, the slowing down occurring in the 16:0-to-20:5 pathway is expected to lead to an over-accumulation of 16:0-CoA. Excess 16:0-CoA can be stored into TAG, via the DGAT/Kennedy pathway. **(H)** An important assumption for this model is that a positive feedback is exerted on the plastid, exporting most its FAs, and explaining that the omega pathway is activated to provide sufficient 20:5 to plastid glycerolipids to compensate the lack of 16-carbon substrates. AAS, acyl-ACP synthetase; ACS, acyl-CoA synthetase; DGAT, diacylglycerol acyltransferase; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; FAD, fatty acid desaturase; MGDG, monogalactosyldiacylglycerol; PAD, palmitoyl ACP desaturase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

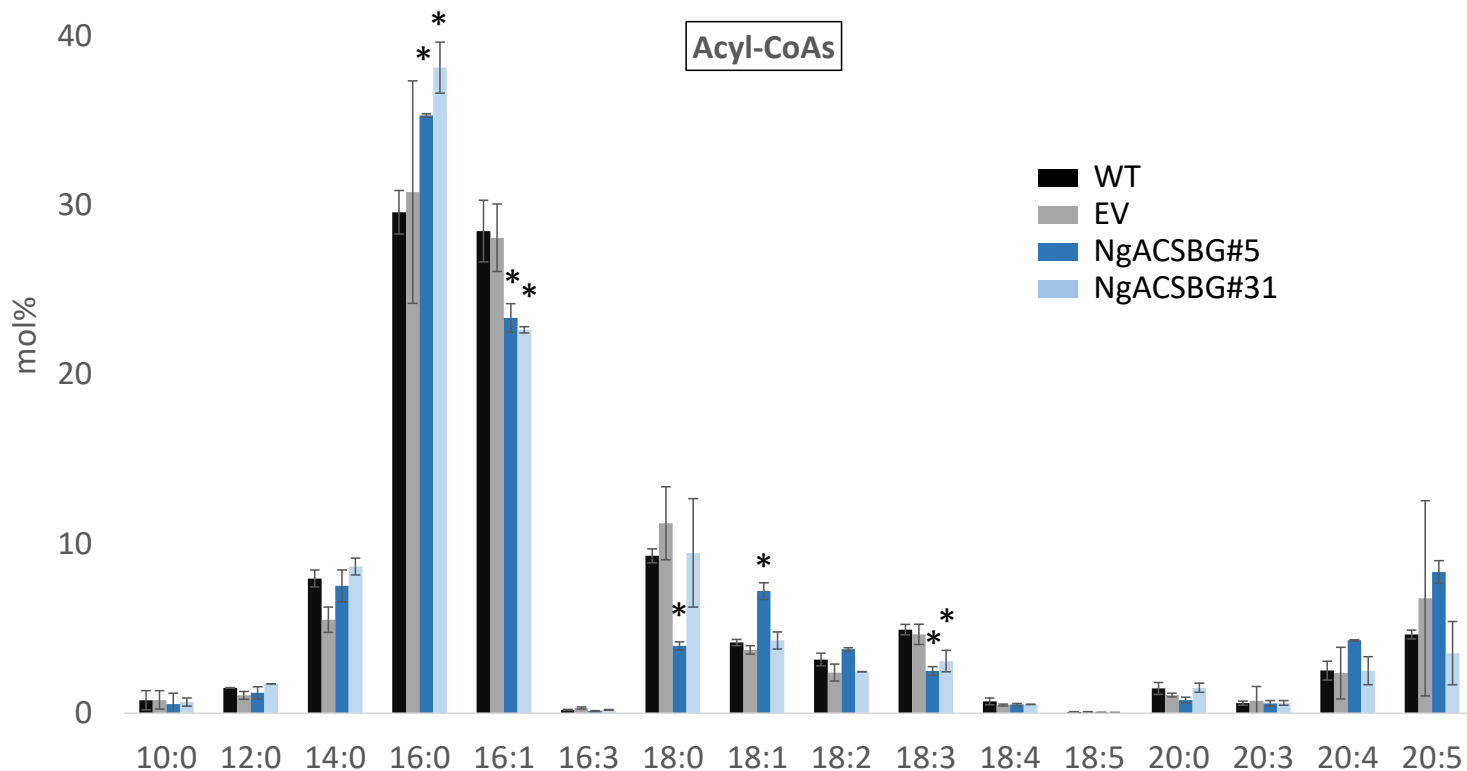


Figure 8: Comparison of the acyl-CoA profiles in MgACSBG#5, MgACSBG#31, untransformed wild type cells (WT) and cells transformed with an empty vector (EV). Cells were cultivated in nutrient replete conditions as described in the Method section (D3). Mass-spectrometry based analysis and data processing were performed by Rothamsted Research, UK. Data are the average of 2 replicates. Error bars, standard deviation. (*), P-value < 0.05; student's t-test using WT as a reference.

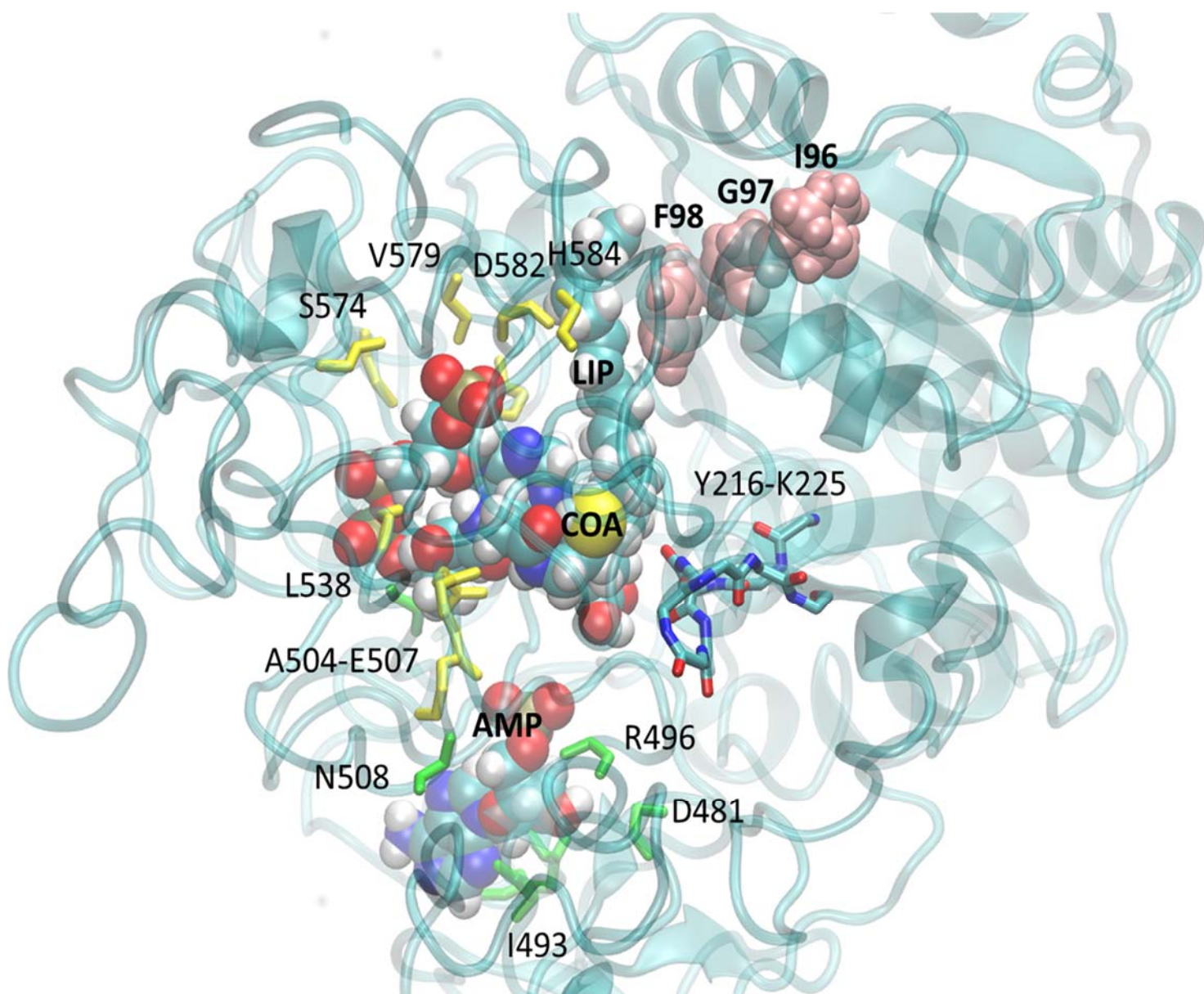


Figure 9. 3D model of MgACSBG bound to CoA, AMP and α -linolenic acid (18:3). Four models of MgACSBG were obtained based on similarity with acetyl- and acyl-CoA synthetase of known 3D structure, as described in the method section. The Mcons1 model of MgACSBG is shown with the visualization program VMD (Humphrey et al., 1996). The backbone of residues around AMP and CoA, listed in Supplemental Table S2A and S2B, are shown in licorice. The IGF triad is represented in pink (I96, G97, F98). The conserved Y216-K225 active site is shown in licorice mode. The position of IGF close to 18:3 is similar in all four MgACSBG models. Abbreviations: COA, Coenzyme A; AMP, adenosine monophosphate; LIP, α -linolenic acid.

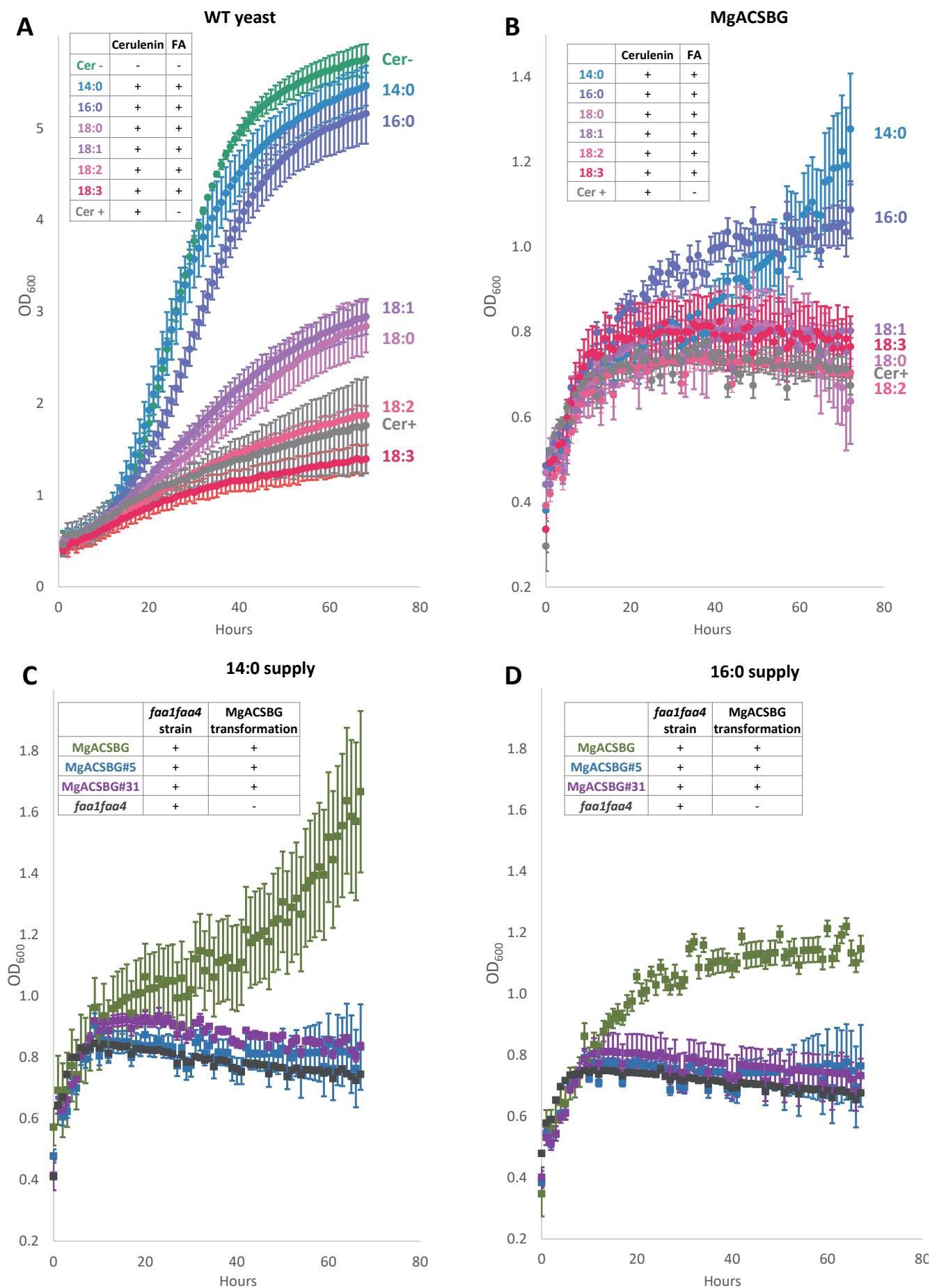


Figure 10. Growth complementation of yeast mutant defective in acyl-CoA activity by MgACSBG variants. (A) WT yeast strain growth curves in galactose medium (Cer-, green circles, positive control), with the addition of 22.5 μ M cerulenin only (Cer+, grey circles, negative control), or with the addition of 22.5 μ M cerulenin and 100 μ M of the following free fatty acids, 14:0, 16:0, 18:0, 18:1, 18:2 and 18:3 (curves ranging from blue to red). (B) Growth curves of MgACSBG complemented *faa1* Δ *faa4* Δ double yeast mutant in galactose medium supplemented by 22.5 μ M cerulenin and 100 μ M of FA (curves ranging from blue to red). Medium with 22.5 μ M cerulenin but FA is used as a negative control (Cer+, grey circles). Average (\pm standard deviation) of biological (n = 3, independent clones) as well as technical (n = 3) replicates are presented. (C) and (D) Growth curves of the *faa1* Δ *faa4* Δ double mutant complemented with MgACSBG (green squares), MgACSBG#5 (blue squares), and MgACSBG#31 (purple squares) in presence of 22.5 μ M cerulenin and 100 μ M 14:0 (C) or 16:0 (D). The *faa1* Δ *faa4* Δ double mutant without complementation is used as negative control and represented by grey circles. Average (\pm standard deviation) of biological (n = 3, independent clones) replicates are presented.

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