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# 1 Targeting TOR signaling for enhanced lipid productivity in algae

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## 11 12 13 **Abstract**

14  
15 Microalgae can be used for many applications, including the development of  
16 third generation biofuels. However, the trade-off between biomass production and lipid  
17 accumulation and productivity still impairs the viable production of sustainable biofuel  
18 from microalgae because neutral lipids accumulate under stress conditions. Recently,  
19 in the model marine diatom *Phaeodactylum tricornutum*, it was shown that inhibiting the  
20 target of rapamycin (TOR) kinase using specific ATP-competitive inhibitors could  
21 overcome this issue. We believe that basic knowledge in this rather new field is  
22 required to develop innovative strategies to improve neutral lipid accumulation in  
23 oleaginous microalgae. In this minireview, we therefore focused on the current  
24 research on the TOR signaling pathway with a focus on its control of lipid homeostasis.  
25 Since TOR has been well-studied in animals, we first describe the effectors of TOR in  
26 animal lipogenesis, adipogenesis and lipolysis. We then propose a tentative model of  
27 TOR regulation of TAG accumulation in algae, a process that has hitherto been  
28 overlooked.

### 29 30 31 **1. Importance of lipids in 3<sup>rd</sup> generation biofuels from microalgae**

32  
33 Lipids, together with carbohydrates, proteins and nucleic acids are essential  
34 components of living cells. While glycerophospholipids, sphingolipids, glycolipids and  
35 sterol lipids are the main components of cell membranes and can also function as  
36 signal transducers, triacylglycerols (TAG) constitute a universal form of transient energy  
37 storage in the form of fatty acids esterified with glycerol. They are found for instance in  
38 the core of lipid droplets in mammalian adipose tissues, in plant seeds, oleaginous  
39 yeasts and microalgae. They are the most abundant neutral lipid species in oils of plant  
40 origin. Stored TAGs can fuel the cell in times of nutrient limitation. The pathway leading  
41 to neutral lipid synthesis is fairly well conserved among eukaryotes: acetyl-CoA derived  
42 from carbon-metabolism is the substrate for *de novo* fatty acid (FA) synthesis, FAs are

43 then conjugated to existing lipophilic alcohols through a series of acylation reactions in  
44 which diacylglycerol (DAG) is the last intermediate before TAG [1]. A particularity of  
45 plant and algae is that the chloroplast is the major site of *de novo* FA synthesis [2].  
46 Cellular TAGs and other neutral storage lipids such as steryl esters are stored in lipid  
47 droplets, and their content can vary considerably depending on cell type and external  
48 environmental cues. For example, plants store TAGs in seeds in order to support the  
49 germination and vegetative growth of the next generation, while microalgae and yeast  
50 accumulate TAGs during starvation.

51 In the modern world the increasing energy demand from human activities  
52 threatens the availability of finite natural resources and the environment due to the  
53 release of CO<sub>2</sub> and other pollutants. Against this background, neutral lipids represent a  
54 promising renewable resource for the biofuel industry, as well as for human and animal  
55 nutrition and health. Oleaginous microorganisms, including certain microalgae, yeasts  
56 and bacteria, can accumulate up to 80% of their biomass in the form of TAGs under  
57 appropriate conditions [3, 4]. These organisms therefore represent a major resource for  
58 the development of third generation biofuels. Algae are a polyphyletic group of  
59 photosynthetic organisms that evolved from multiple endosymbiotic events. The  
60 endosymbiosis of a cyanobacterium by a non-photosynthetic eukaryote led to the  
61 Archaeplastida group of chloroplast-containing eukaryotes which includes red algae,  
62 glaucophytes, green algae and land plants. Further endosymbiosis of green or red  
63 algae by other non-photosynthetic eukaryotes led to the evolution of diverse groups of  
64 algae including euglenoids and diatoms [5]. Illustrating this diversity, diatoms have  
65 retained unique characteristics from different domains of life [6] such as the presence  
66 of the urea cycle [7], which is typical of animals, and a sterol biosynthetic pathway that  
67 uses intermediates from both plant and fungal sterol biosynthesis [8]. The use of  
68 microalgae for lipid stock production has many advantages that have been widely  
69 explored and described [9]: a broad range of microalgal strains can be exploited for oil  
70 production [4, 10]; they can reach high growth rates (1 to 4 divisions per day for the  
71 model green alga, *Chlamydomonas reinhardtii* [11]); and by consuming CO<sub>2</sub> they  
72 contribute to reducing greenhouse gas emissions. Moreover, because microalgae can  
73 use a variety of other inorganic nutrients they can even be exploited for bio-remediation  
74 from wastewaters [3]. Since microalgae are able to grow in controlled environments like  
75 that of photobioreactors as well as under extreme conditions (high salinity, and across  
76 a broad range of temperature and light intensities), they are not subject to seasonal  
77 changes and do not necessarily compete with crops for land and water.

78 Microalgae typically produce large amounts of lipids under stress conditions  
79 such as nutrient limitation, and most notably under nitrogen (N) starvation. However,  
80 these stresses interrupt cell proliferation and therefore reduce total biomass and limit  
81 the overall lipid yield. In the last decade there has been a gold rush for the selection of  
82 strains or the creation of engineered microorganisms able to accumulate more lipids. In  
83 this regard a path frequently beaten by researchers is to knockout or overexpress  
84 genes involved in carbon and lipid metabolism [10]. However, the growth of these

85 strains is often compromised and a better knowledge of the regulation of the balance  
86 between growth and lipid accumulation is still required. In this context, a new route is  
87 represented by the study of the TOR (Target of Rapamycin) kinase pathway [12], which  
88 is a central hub in the regulation of various cell cycle processes including the regulation  
89 of nutrient and energy homeostasis, lipid synthesis and is conserved in eukaryotes [13].  
90 We will first present evidence for the importance of TOR signaling in the regulation of  
91 lipid homeostasis from the wealth of studies in animals, and then we will present how  
92 manipulation of TOR signaling can improve TAG productivity in algae. Finally, we will  
93 discuss how recent studies in photosynthetic eukaryotes, in parallel with discoveries on  
94 TOR signaling in animals, can be used to understand how TOR regulates TAG  
95 accumulation in algae and, ultimately, to target TOR signaling for increased lipid  
96 productivity.

## 97 98 99 **2. The principal TOR signaling effectors that control lipid metabolism in** 100 **animals**

101  
102 TOR (Target of Rapamycin) is a large (280 kDa) multi-domain kinase belonging  
103 to the phosphatidylinositol kinase-related kinase (PIKK) family. TOR is inhibited by  
104 rapamycin (Fig. 1) through interaction with the 12 kDa TOR-FRB domain (FKBP12-  
105 rapamycin-binding domain). Two canonical TOR containing complexes (TORC),  
106 TORC1 and TORC2, exist in both mammals and yeast. Recently, different forms of a  
107 TORC3 that differ in structure, function and subcellular localization have been  
108 discovered in mammals [14-17]. The TORC1 core, which is composed of the TOR  
109 kinase, RAPTOR (regulatory associated protein of TOR) and LST8 (lethal with SEC13  
110 protein 8), is found in all eukaryotes [18] and is sensitive to the allosteric inhibitor  
111 rapamycin, which binds to the FRB domain of TOR. TORC1 is regulated by nutrients  
112 such as glucose, amino acids, as well as by growth factors and is inhibited by stress-  
113 related signals such as starvation and hypoxia. Conserved functions of TORC1 include  
114 the activation of protein, lipid and nucleotide synthesis, as well as promoting  
115 progression of the cell cycle. At the same time, TORC1 also inhibits stress-responsive  
116 genes and catabolic processes like autophagy [19, 20]. Upstream signals are funneled  
117 towards TORC1 via different repressors and activators that regulate TOR intracellular  
118 localization and activity. One of the major upstream repressors of TORC1 is the AMP-  
119 activated protein kinase (AMPK), a major sensor of cellular energy status via the  
120 AMP/ATP and ADP/ATP ratio in mammals [21]. The orthologues of AMPK in yeast  
121 (SNF1, sucrose non fermenting 1) and plants (SnRK1, SNF1-related kinase 1) [22, 23]  
122 are not responsive to AMP, indicating that there are distinct strategies for energy  
123 sensing in different organisms.

124 As a general rule, active TOR in mammals promotes lipogenesis and  
125 adipogenesis and inhibits lipolysis [24]. Lipid synthesis and storage are promoted by

126 the SREBP (sterol regulatory element-binding protein) transcription factor, a master  
127 regulator of the transcription of lipo- and sterolgenic genes such as fatty acid synthase,  
128 acetyl-CoA carboxylase alpha, stearoyl CoA-desaturase, HMG-CoA reductase, and  
129 farnesyl diphosphate synthase [13]. SREBP is retained in the membrane of the  
130 endoplasmic reticulum where it is unable to activate transcription. In response to sterol  
131 depletion SREBP is cleaved and the SREBP DNA-binding domain is imported into the  
132 nucleus where it promotes the transcription of lipogenic enzymes [25]. In mammals,  
133 TORC1 regulates the activation of SREBP via two main mechanisms: the  
134 phosphorylation of (i) the ribosomal S6 kinase 1 (S6K1) to promote lipogenic processes  
135 including the processing of SREBP, and (ii) of lipin-1, a phosphatidic acid phosphatase  
136 (PAH) that converts phosphatidic acid (PA) into DAG, an important precursor of  
137 membrane and neutral lipids. Upon TORC1 inhibition, lipin-1 becomes  
138 dephosphorylated and is imported into the nucleus where it inhibits SREBP to down-  
139 regulate lipid synthesis. Interestingly, TORC stability and activity is itself dependent on  
140 PA, which binds the FRB domain of TOR and acts as an indicator of nutrient sufficiency  
141 [26, 27]. Another S6K1 target, the Serine/Arginine (SR)-rich protein kinase 2 (SRPK2),  
142 which controls the expression of lipogenic enzymes was recently reported [28]. It  
143 mediates the phosphorylation of SR proteins and their interaction with the spliceosomal  
144 protein U1-70K, which is responsible for the splicing of lipogenic pre-mRNAs. The  
145 phosphorylation of another mTORC1-S6K target, the glutamylprolyl tRNA synthetase  
146 (EPRS), induces EPRS release from the amino acyl tRNA multisynthetase complex  
147 and its interaction with the fatty acid transport protein 1, promoting its translocation to  
148 the plasma membrane and importing fatty acid to the cells [29]. In addition to inducing  
149 lipogenesis and controlling cellular lipid homeostasis in different cell types in the brain  
150 and liver, mammalian TORC1 also has a positive effect on the differentiation of stem  
151 cells into mature adipocytes and in the formation of adipose tissue [30]. TORC1  
152 controls lipid metabolism in white and brown adipose tissues that respectively favor  
153 lipid storage or lipid use. The mobilization of the energy stored in the form of TAG,  
154 through the action of several lipases [31] and  $\beta$ -oxidation of FA can generate either  
155 ATP or heat through the uncoupling protein 1 (UCP1) that decouples the mitochondrial  
156 electrochemical gradient. These processes, which are still under study, illustrate the  
157 fundamental role of TOR in regulating lipid and energy metabolism at the cell and  
158 organism level.

159

### 160 **3. TOR inhibition can induce a “get fat growth” regime in algae**

161

162 Despite the large diversity and the complex evolution of photosynthetic  
163 eukaryotes, all plants and algae investigated contain a conserved TORC1 with at least  
164 TOR, RAPTOR and LST8 [32]. No other TOR complexes have yet been described in  
165 these groups of organisms. TOR is an essential regulator of proliferation in both plants  
166 and algae as shown by the embryo-lethal phenotype of *tor* null mutants in plants, and

167 the repression of both plant and algal growth by the allosteric TOR inhibitor rapamycin  
168 or by ATP-competitive TOR inhibitors [33-36].

169 Two independent studies conducted in plants and algae demonstrated the link  
170 between TOR repression and neutral lipid accumulation [37, 38]. Repression of *tor*  
171 expression by inducible RNA silencing in *Arabidopsis* seedlings led to major metabolic  
172 changes including the massive accumulation of storage lipids and starch [37].  
173 Likewise, repression of TOR activity by rapamycin treatment led to increased levels in  
174 TAG synthesis-related genes, such as glycerol-3-phosphate acyltransferase and acyl-  
175 CoA:diacylglycerol acyltransferase (DGAT), and consequently to TAG accumulation in  
176 *Cyanidioschyzon merolae* (red algae) and *Chlamydomonas reinhardtii* (green algae)  
177 [39]. These studies were pioneering in that they opened the way to targeting TOR  
178 signaling for improving plant and algal lipid production. More recent studies conducted  
179 on other diverse groups of microalgae, including the diatom *Phaeodactylum tricornutum*  
180 and the euglenoid *Euglena gracilis*, showed that TOR inhibition results in the  
181 accumulation of large lipid droplets rather similar to those that appear in response to N  
182 starvation [34, 39-42] (Fig. 2). TOR inhibition also induces lipid droplet accumulation in  
183 budding yeast, suggesting that this phenotype might be a common feature rather than  
184 being specific to photosynthetic eukaryotes [40]. Although TAG accumulation seems to  
185 be a general response to TOR inhibition in algae and plants, other associated  
186 phenotypes are not always similar. For example, while we observed that *P. tricornutum*  
187 treatment with the ATP-competitive TOR inhibitor AZD-8055 (Fig. 1) could lead to a  
188 stronger decrease in chlorophyll fluorescence than N starvation, no significant effect of  
189 rapamycin on chlorophyll content was observed in *C. reinhardtii* by Roustan and  
190 Weckwerth [43]. However, while ATP-competitive TOR inhibitors can efficiently inhibit  
191 TOR, rapamycin is less efficient, at least in animals where it only partially inhibits TOR  
192 function [44]. This is probably also the case in algae since rapamycin, even at high  
193 concentrations, only partially represses algal growth to 40-50% of that of untreated  
194 cells [34, 35, 45, 46]. On the other hand, ATP-competitive TOR inhibitors can almost  
195 completely stop algal proliferation [18, 34, 41]. More comparative analyses with similar  
196 TOR inhibitors and at similar effective doses are therefore required to accurately  
197 compare the extent and diversity of TOR functions in different algae.

198 As discussed above, a major bottleneck in the development of algae for biofuel  
199 production is the fact that most conditions that induce TAG accumulation also repress  
200 growth, which strongly limits productivity. Obviously, this is also a concern with TOR  
201 inhibitors considering the essential and conserved positive function of TOR in the  
202 regulation of cell growth and proliferation. However, analysis of the dose-dependent  
203 effects of AZD-8055 in the diatom *P. tricornutum* allowed the identification of a dose  
204 that induced a 3 to 4 fold higher lipid productivity than N starvation. This enhanced lipid  
205 productivity was due to a moderated impairment of proliferation that allowed cells to  
206 grow and divide while accumulating TAG, in what was coined a “get fat-growth regime”  
207 [34]. Notably, it is not possible to titrate N to achieve the same effect, because N will be  
208 rapidly consumed by the cells. These results show that fine tuning of TOR activity can

209 improve lipid productivity, and underline the need to fully decipher the TOR signaling  
210 axis involved in lipid homeostasis to improve the balance between proliferation and  
211 TAG accumulation.

212  
213

#### 214 **4. How could the TOR signaling pathway regulate neutral lipid accumulation** 215 **in algae?**

216

217 Recent phosphoproteomics and pharmacogenetics analyses have identified  
218 potential members of the TOR pathway in photosynthetic eukaryotes. Although some of  
219 these data were obtained in *Arabidopsis* and have not yet been confirmed in  
220 *Chlamydomonas* or other more distantly related algae, this section presents an  
221 overview of the potentially conserved TOR signaling axis involved in lipid homeostasis,  
222 and a tentative model of how TOR might regulate TAG accumulation in algae (Fig. 3).

223 S6Ks are conserved TOR targets found in plants and algae that are known to  
224 regulate translation [47, 48]. Phosphoproteomic studies of *Chlamydomonas* cells  
225 treated with rapamycin or AZD-8055 identified TOR-dependent phosphorylation sites in  
226 S6K and ribosomal S6 protein [43, 49]. In mammals, S6K regulates SREBP processing  
227 and other lipogenic genes. However, SREBP homologs are not found in plants and  
228 algae. Interestingly, one of the two *Arabidopsis* S6Ks localizes to the nucleus,  
229 suggesting its potential involvement in transcriptional regulation [50]. Orthologues of  
230 the SREBP repressor and PAH (lipin-1) are also found in plants and algae [51].  
231 *Arabidopsis* has two PAHs (PAH1 and 2), whose loss of function induces increase in  
232 phospholipid content [51]. In algae species, transcriptomics, proteomics or  
233 metabolomics did not report PAHs or PA but this might reflect that such signaling  
234 proteins and PA are hard to detect in the nutrient conditions devoted to increase  
235 cellular lipid contents [52]. In the green microalga *Chlorella vulgaris*, proteomics studies  
236 reported that S6K increased under nutrient depleted conditions [53] and the  
237 phosphatidic acid phosphatase PAP was identified [54]. Strikingly, PAH2 is  
238 phosphorylated in a TOR dependent manner in *Arabidopsis*, suggesting a conservation  
239 of the link between TOR and PAH in photosynthetic eukaryotes [55]. PAH1 is also  
240 phosphorylated by the cyclin-dependent kinases (CDKs) in *Arabidopsis* suggesting a  
241 further link between lipid homeostasis and cell cycle progression [56]. Hence, although  
242 a direct link between S6K/PAH and TAG accumulation has not yet been demonstrated  
243 in algae, their conservation strongly suggests that they may play a significant role in  
244 algal lipid homeostasis (Fig. 3).

245 Other potential effectors of TOR signaling were directly identified in genetic  
246 screens on algae. First, two members of the DYRK (dual-specificity tyrosine-  
247 phosphorylation regulated kinase) family of protein kinases were isolated in screens for  
248 *Chlamydomonas* insertional mutants defective for oil and starch accumulation [57, 58].  
249 One, named TAR1 (triacylglycerol accumulation regulator 1) is the homolog of the

250 yeast and *Arabidopsis* YAK1 (yet another kinase 1), and the other, named DYRKP,  
251 belongs to a plant and algal DYRK subfamily. A link between YAK1/DYRK and TOR  
252 signaling was revealed recently in plants during a screen for *Arabidopsis* mutants  
253 resistant to the TOR inhibitor AZD-8055. *Arabidopsis* YAK1 is a negative regulator of  
254 growth that is repressed by TOR [59], interacts with TOR, and is phosphorylated in a  
255 TOR-dependent manner [55] but it is not yet clear how TOR-YAK/DYRK signaling  
256 affects TAG levels in algae. Another screen aimed at identifying TOR signaling  
257 effectors in algae led to the identification of the rapamycin-hypersensitive mutant *vip1-1*  
258 [60]. VIP1 encodes an inositol hexakisphosphate kinase that pyrophosphorylates the  
259 inositol phosphate InsP6 to produce the signaling molecules InsP7 and InsP8. The fact  
260 that InsP7 and InsP8 levels drop in rapamycin-treated wild-type cells confirmed a link  
261 with TOR signaling [60]. Interestingly, the higher levels of TAGs in the *vip1-1* mutant led  
262 to the conclusion that InsP7 and InsP8 activate growth at the expense of TAG  
263 accumulation [60]. In *Arabidopsis*, AZD-8055 leads to increased phosphorylation of the  
264 VIP1 homolog VIP2 suggesting an indirect regulation by TOR [55]. Interestingly, both  
265 *tar1* and *vip1* mutant phenotypes are dependent on the presence of a carbon source.  
266 *tar1* mutant fails to accumulate TAGs when N starvation is coupled to acetate supply  
267 (photomixotrophy) but accumulates high level of TAGs in photoautotrophic N-deficient  
268 conditions [61]. Similarly, *vip1* is hypersensitive to TOR inhibitors on acetate supplied  
269 media but not in photoautotrophic conditions [60]. This highlights the importance of the  
270 TOR signaling pathway in integrating different nutrient signals to regulate growth and  
271 reserve accumulation in algae.

272 Last but not least, autophagy was the first intracellular process connected to  
273 TOR signaling to be reported in algae. Rapamycin treatment was shown to induce  
274 vacuolization [35] and post-translational modification of the key autophagy-related  
275 protein ATG8 in *C. reinhardtii* [62]. More recently, ATG7 phosphorylation was shown to  
276 be dependent on TOR [46], suggesting that TOR may regulate autophagy via this  
277 protein. In addition, a recent report showed that treatment of cells with concanamycin  
278 A, an inhibitor of the autophagic flux, repressed TAG accumulation in response to N  
279 starvation. This work indicates that autophagy and vacuolar function are therefore  
280 required for lipid droplet accumulation in *Chlamydomonas* [63]. It is therefore likely that  
281 the activation of autophagy plays an important role in lipid droplet accumulation in  
282 response to TOR inhibition.

283  
284

## 285 **Perspectives**

286

287 This minireview shows the importance of measuring proliferation and/or growth  
288 in connection with TAG productivity whatever the context. Manipulation of specific TOR  
289 signaling axis and the use of different ATP competitive inhibitors of TOR, at different  
290 concentrations, may help to achieve a better TAG productivity. Identifying the



291 downstream targets of TOR needs further investigations to decipher the top secret of  
292 TOR in different organisms and especially in algae that are still overlooked for TOR  
293 function. More comparative studies are indeed required, particularly between different  
294 algae due to their diversity and because most TOR effectors were studied in the model  
295 green alga *Chlamydomonas reinhardtii*, which is not necessarily the best choice for  
296 biotechnological applications. It is obvious that there are still many remaining questions  
297 to understand the relationships between TOR and TAG accumulation in algae and  
298 many studies to perform in that fascinating field.

299

300

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## 484 **Figure legends**

485

486 **Fig. 1.** Chemical structures of the TOR inhibitors rapamycin (A) and AZD-8055 (B).

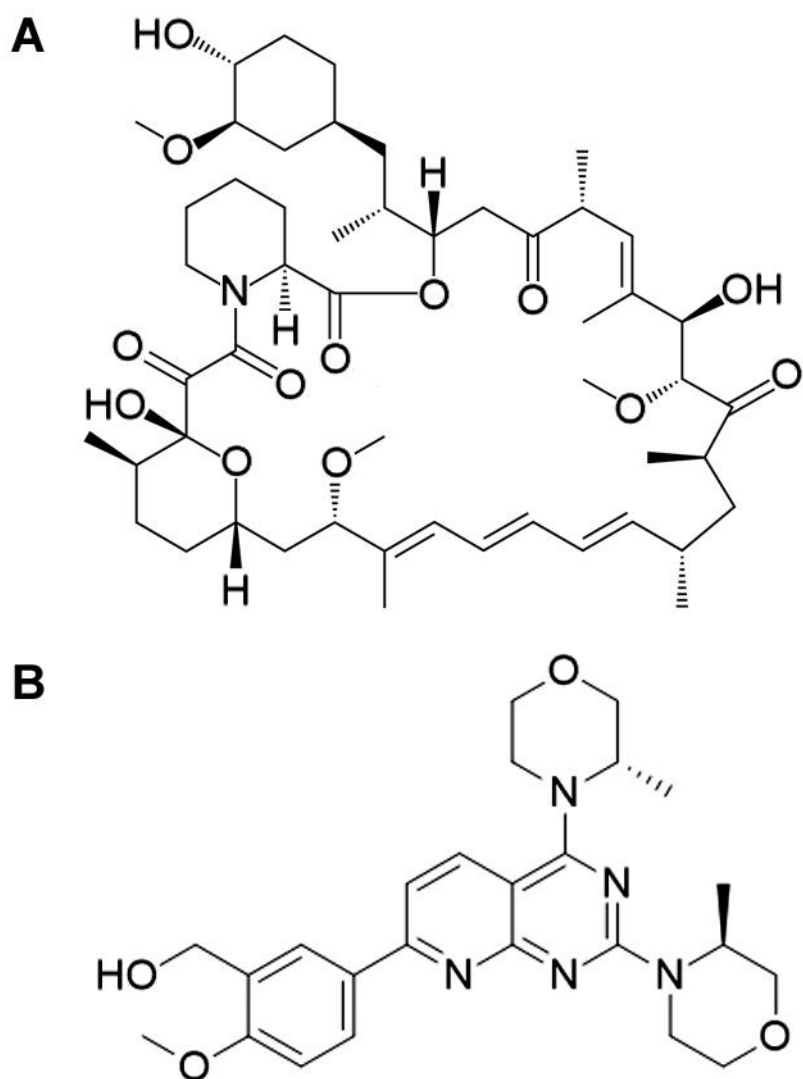
487

488 **Fig. 2.** Effect of TOR inhibition on lipid storage in microalgae. A) Nile Red staining of  
489 lipid droplets in *Phaeodactylum tricornutum* after five days of treatment with DMSO  
490 (left) or AZD-8055 2  $\mu$ M (right), scale bars 10  $\mu$ m (modified with permission from  
491 Prioretti et al. 2017). B) BODIPY and AC-202 merged staining of lipid droplets in  
492 *Chlamydomonas reinhardtii* after 48h of treatment with DMSO (left) or AZD-8055 2  $\mu$ M  
493 (right), scale bar 5  $\mu$ m (modified from Harchouni et al. 2018). C) BODIPY staining of  
494 lipid droplets in a rapamycin-sensitive strain of *Cyanidioschyzon merolae* expressing

495 yeast FKBP12 after 48h of treatment with DMSO (left) or rapamycin 1  $\mu$ M (right), scale  
496 bars 10  $\mu$ m (modified with permission from Imamura et al. 2015). D) Nile Red staining  
497 of lipid droplets in *Euglena gracilis* after five days of treatment with ethanol (left) or  
498 rapamycin 1  $\mu$ M (right), scale bars 20  $\mu$ m (modified with permission from Mukaida et al.  
499 2016).

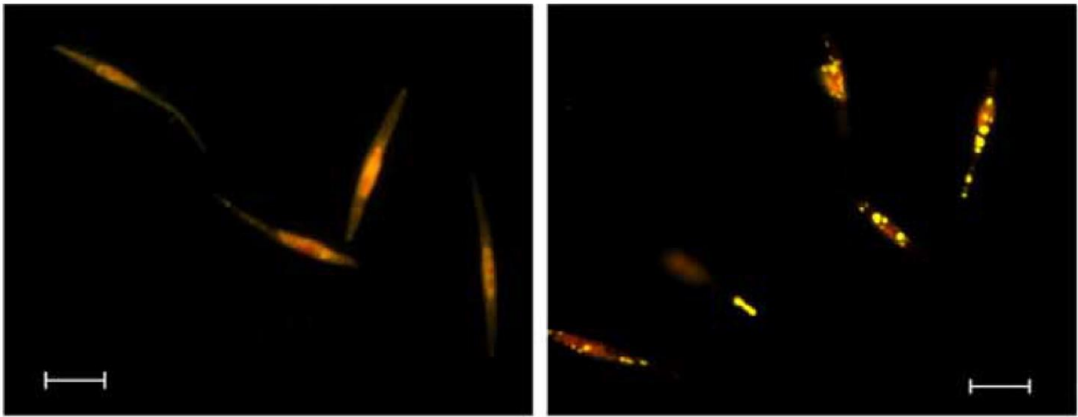
500  
501 **Fig. 3.** Model of effectors of TOR-dependent regulation of growth and lipid homeostasis  
502 in microalgae.

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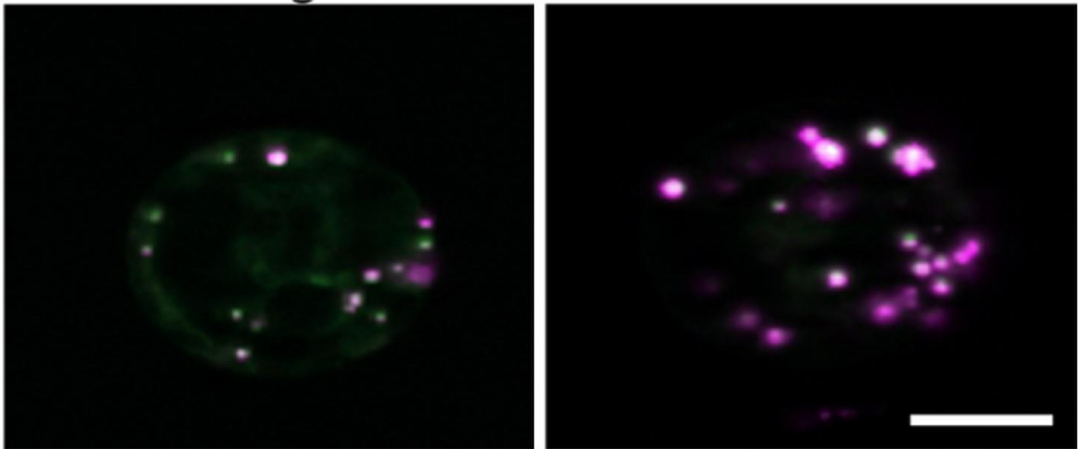


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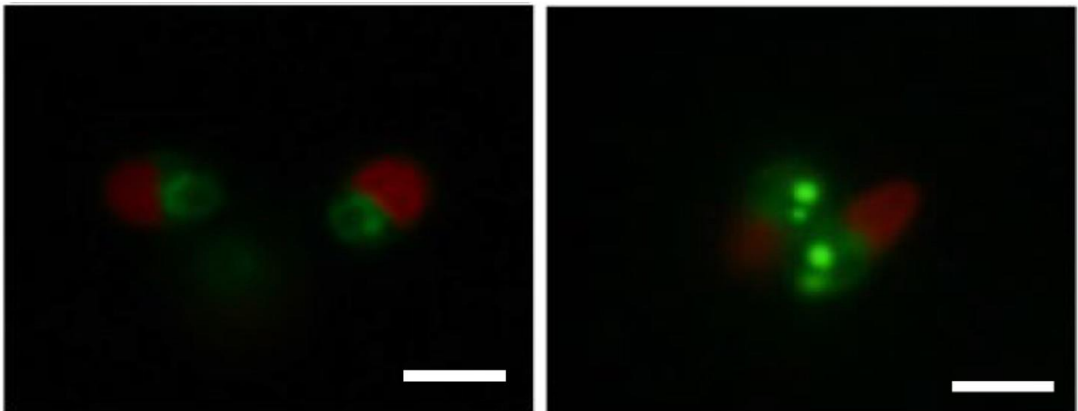
**A**



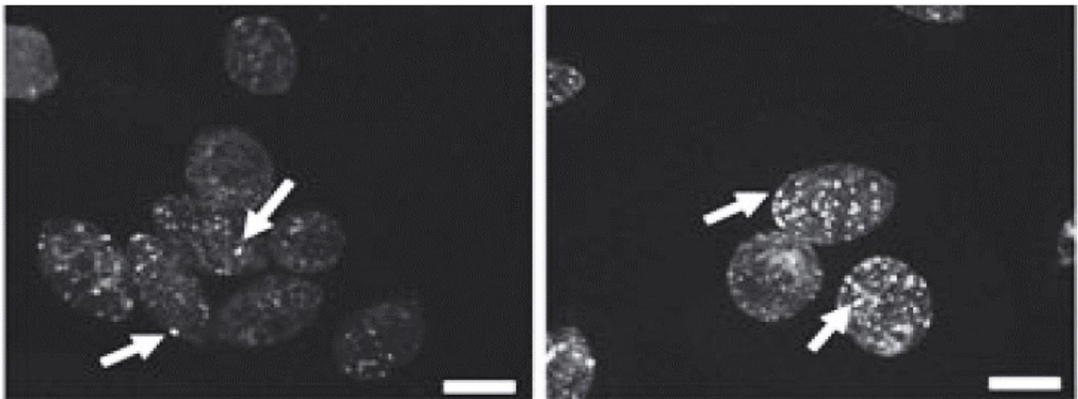
**B**

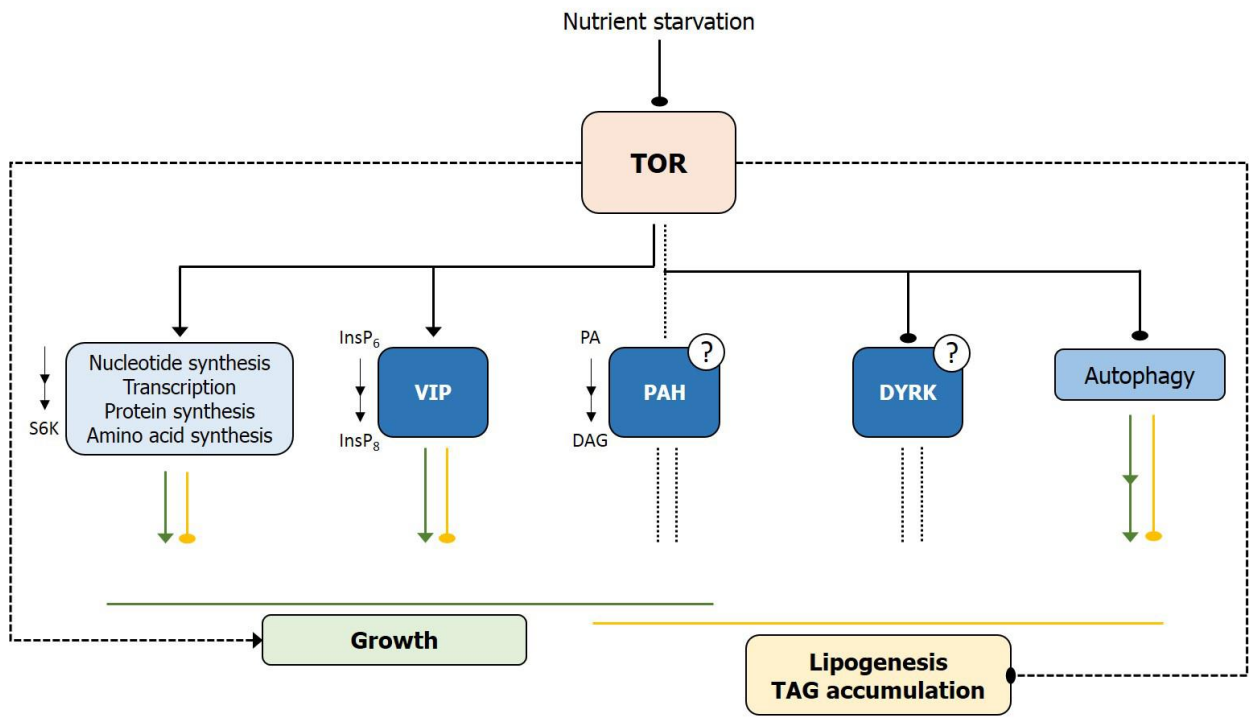


**C**



**D**





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