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1 **Short title:**

2 **Acyl-lipid and sterol metabolisms in diatoms**

4 **Title:**

5 **Relationship between acyl-lipid and sterol metabolisms in diatoms**

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18 E.M. and J.L. contributed to the writing of the article.

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20 Diatoms, *Phaeodactylum*, Triacylglycerol, Sterol, **Acyl-lipid**

Abstract

1 Diatoms are a phylum of unicellular photosynthetic eukaryotes living in oceans and fresh
2 waters, characterized by the complexity of their plastid, resulting from a secondary
3 endosymbiosis event. In the model diatom *Phaeodactylum tricornutum*, fatty acids (FAs) are
4 synthesized from acetyl-CoA in the stroma of the plastid, producing palmitic acid. FAs are
5 elongated and desaturated to form very-long chain polyunsaturated fatty acids (VLC-PUFAs)
6 in domains of the endomembrane system that need to be identified. Synthesis of VLC-PUFAs
7 is coupled with their import to the core of the plastid *via* the so-called “omega” pathway. The
8 biosynthesis of sterols in diatoms is likely to be localized in the endoplasmic reticulum as
9 well as using precursors deriving from the mevalonate pathway, using acetyl-CoA as initial
10 substrate. These metabolic modules can be characterized functionally by genetic analyzes or
11 chemical treatments with appropriate inhibitors. Some ‘metabolic modules’ are characterized
12 by a very low level of metabolic intermediates. Since some chemical treatments or genetic
13 perturbation of lipid metabolism induce the accumulation of these intermediates, channeling
14 processes are possibly involved, suggesting that protein-protein interactions might occur
15 between enzymes within large size complexes or metabolons. At the junction of these
16 modules, metabolic intermediates might therefore play dramatic roles in directing carbon
17 fluxes from one direction to another. Here, acetyl-CoA seems determinant in the balance
18 between TAGs and sterols. Future lines of research and potential utilization for
19 biotechnological applications are discussed.

1. Introduction

1
2 Diatoms are major components of the microbial food web in oceans and fresh waters, playing
3 an essential role in the carbon geochemical cycle, as primary producers [1, 2]. They
4 contribute to at least 20% of global primary production (through the fixation of CO₂ by
5 photosynthesis), representing about forty-five gigatons of organic carbon per year [3, 4].
6 Their lifestyle exposes these organisms to permanent environmental variations and stresses,
7 often marked by an imbalance in some elements, such as nitrogen and phosphorus, triggering
8 an accumulation of carbon storing molecules [5-10]. Diatoms store carbon in two forms,
9 triacylglycerols (TAGs) and to some extent chrysolaminarin, a soluble linear polymer of
10 $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linked glucose [11, 12]. TAGs are a class of glycerolipids composed of
11 three fatty acids esterified to a glycerol backbone (for review, [8]). Because of their high
12 hydrophobicity, TAGs generally accumulate inside lipid droplets [9]. TAGs extracted from
13 microalgae, including diatoms, have attracted an increasing interest for multiple
14 biotechnological applications. TAGs enriched in medium-chain fatty acids with little or no
15 unsaturations (or double bonds) are considered a promising feedstock for biofuel production
16 or green chemistry. In contrast, TAGs enriched in very long-chain polyunsaturated fatty acid
17 (VLC-PUFAs such as eicosapentaenoic acid (EPA, 20:5 n-3) or docosahexaenoic acid (DHA,
18 22:6 n-3) are of high interest for nutrition and human health [8]. Microalgae are also
19 interesting for the synthesis of pigments (carotenoids for food, cosmetic and pharmaceutical
20 industries), sterols (for nutraceuticals), proteins and enzymes for the conversion of EPA into
21 DHA (for review, [13]).

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39 The establishment of models, such as the pennate diatom *Phaeodactylum tricornutum* or the
40 centric diatom *Thalassiosira pseudonana*, through genome sequencing [14, 15], development
41 of methods for transformation (biolistics, electroporation or conjugation) [16-19] and genetic
42 engineering (TALEN and CRISPR/Cas9) [20-24], has allowed to address the carbon
43 metabolic pathways in this important clade of microalgae. This mini-review focuses on
44 pathways leading to the synthesis of TAGs and sterols, based on studies conducted mainly in
45 *P. tricornutum*. Most recent studies have highlighted that these two metabolic pathways are in
46 direct competition with respect to carbon, especially at their common point of entry, acetyl-
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2. Overview of fatty acid and glycerolipid metabolism in diatoms

1 Building blocks for glycerolipids consist of glycerol-3-phosphate, providing the three-carbon
2 backbone (termed *sn*-1, *sn*-2 and *sn*-3 carbons) found in all glycerolipids, fatty acids and
3 different types of polar head precursors (for review, [25]). Positions *sn*-1 and *sn*-2 are
4 esterified to fatty acids, whereas position *sn*-3 can be linked either to a polar head or to a third
5 fatty acid. Knowledge on glycerolipid metabolism in diatoms, a secondary endosymbiont, is
6 still incomplete, and mainly based on the translation of what has been shown in primary
7 endosymbionts, such as plants and green algae. Indeed, an important feature of diatoms is **the**
8 **complexity of the plastid**, resulting from a secondary endosymbiosis event, following the
9 engulfment of a red alga within a secondary eukaryotic host [9, 26]). As illustrated in **Figure**
10 **1**, four membranes delineate their plastid ([26, 27]). In addition, the outermost membrane of
11 the plastid (cERM in **Figure 1**) is in direct continuity with the outermost membrane of the
12 nuclear envelope, meaning therefore a contact between the plastid and the endomembrane
13 system [9, 28].
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24 **2.1. Synthesis of fatty acids in the stroma of secondary plastids**

25 Fatty acids are carboxylic acids having an aliphatic chain of variable length (for review, [8])
26 In diatoms, the synthesis of fatty acids occurs in the stroma of the plastid, following the same
27 processes as those deciphered in primary endosymbionts, *i.e.* by the action of a FAS II (Fatty
28 Acid Synthase type II) system. It requires a pool of acetyl-CoA [29].
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33 Acetyl-CoA is a precursor for several metabolic pathways (lipids, proteins, carbohydrates).
34 Acetyl-CoA can be synthesized first from free acetate as a substrate of the Krebs Cycle by an
35 acetyl-CoA synthetase (ACS), thus generating a mitochondrial acetyl-CoA pool [30] **Figure**
36 **2)** Acetyl-CoA can also be synthesized by the oxidative decarboxylation of pyruvate by the
37 action of a plastidial pyruvate dehydrogenase complex (PDH), composed of three enzymes:
38 pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and dihydrolipoyl
39 dehydrogenase (E3) [31]. This route is preferentially used for the synthesis of the acetyl-CoA
40 pool used for fatty acid synthesis in plants and algae [32]. Therefore, pyruvate is also an
41 important substrate for fatty acid synthesis. Pyruvate is synthesized by many metabolic
42 pathways such as glycolysis, the pentose phosphate pathway or by the conversion of malate
43 by the malic enzyme (**Figure 2**, ME). Several studies have been conducted on ME (called
44 respectively PtME and ME1) in *P. tricornutum* [33, 34]. The PDH activity is inhibited by a
45 pyruvate dehydrogenase kinase (PDK). Antisense knockdown of PDK in *P. tricornutum*
46 (PtPDK) was shown responsible for the accumulation of neutral lipids raising the importance
47 of mitochondrial acetyl-CoA pool in diatoms [35]. Transcriptomic analyzes carried out in *P.*
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1 *tricornutum* in the presence of 3 and 10 μM of nitric oxide [6] or 10 and 20 μM of
2 ethynylestradiol [7] (See 5) show an increase in the expression of the E2 (Plastid-DHLATa:
3 Phatr3_J23850 and Plastid-DHLATb: Phatr3_EG02309) and E3 (Plastid-LPD:
4 Phatr3_J30113) subunits of the PDH.
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6 Acetyl-CoA can also be produced by the degradation of fatty acids during β -oxidation and by
7 disproportionation of pyruvate to acetyl-CoA and formate by a pyruvate formate lyase (PFL).
8 A gene coding for this enzyme has been found in the centric diatom *T. pseudonana*
9 (THAPSDRAFT_36689) but not in other Stramenopiles [36] (Figure 2).
10

11 In the stroma, acetyl-CoA is converted into malonyl-CoA by an acetyl-CoA carboxylase
12 (ACCase) adding a carboxyl group (Figure 2). Metazoa present a unique ACCase homomeric
13 form localized in the cytoplasm and the mitochondria whereas plants and algae, making up
14 the Viridiplantae superclade, have a homomeric and heteromeric ACCase forms localized in
15 the plastid (ACC1) and cytoplasm (ACC2) [13]. Several genetic studies have been conducted
16 in diatoms to better characterize this enzyme. The overexpression of plastid ACCase in the
17 diatoms *Cyclotella cryptica* and *Navicula sapuvila* leads to an increase in enzymatic activity
18 but does not modify the amount of lipids [37, 38]. Opposite results were obtained in *P.*
19 *tricornutum*. The plastid ACCase (PtACC-1) of *P. tricornutum*, overexpressed in *Escherichia*
20 *coli*, led to an increase in enzymatic activity of 1.72 fold, a 2-fold increase in the amount of
21 neutral lipid determined by Nile red staining and an increase of 13% in monounsaturated fatty
22 acids synthesis [39].
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24 Then, malonyl-CoA is converted into malonyl-acyl carrier protein (malonyl-ACP) by a
25 malonyl-CoA:acyl-carrier protein transacylase (MCAT). From malonyl-ACP, a series of four
26 enzymatic reactions transfer two carbons per elongation cycle. The first reaction, catalyzed by
27 a β -ketoacyl-ACP synthase III (KASIII), consists in the condensation of an acetyl-CoA with a
28 malonyl-ACP, forming after decarboxylation of a molecule of 3-ketoacyl-ACP. Three genes
29 are predicted to encode ketoacyl-ACP synthases in *P. tricornutum* (Plastid-KASa,
30 Phatr3_J37367; Plastid-KASb, Phatr3_J52648 and Organelle-KAS, Phatr3_J18940). 3-
31 ketoacyl-ACP is then reduced into 3-hydroxyacyl-ACP by a 3-ketoacyl-ACP-reductase,
32 NADPH-dependent (KAR, Phatr3_J13073). The last two reactions consist in the synthesis of
33 an acyl group, by the stepwise action of an hydroxyacyl-ACP dehydratase (HAD,
34 Phatr3_J55157) forming 2,3-transenoyl-ACP, and of an enoyl-ACP reductase (ER,
35 Phatr3_J10068). The final acyl group is thio-esterified to an acyl carrier protein by an ACP
36 acyltransferase. It has been proposed that the FAS II system was the main machinery
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1 synthesizing fatty acids in diatoms [40], resulting in the formation of 16:0-ACP after seven
2 cycles [41, 42]. It remains unclear whether diatom FASII could form 18:0-ACP as in plants.

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4 Subsequently, acyl-ACPs are likely to follow two directions, i) an incorporation inside the
5 plastid *via* the so-called "prokaryotic" pathway (see 2.2) or ii) an export outside the plastid to
6 the cytosol, where elongation/desaturation processes occur leading to the production of VLC-
7 PUFAs [42-44] (see below). In the endoplasmic reticulum (ER), fatty acids can serve for the
8 synthesis of glycerolipids *via* the so-called "eukaryotic" pathway [45, 46] (see 2.3). It is
9 considered that the export of fatty acids outside the plastid requires the release of free fatty
10 acids (FFA), by the action of an acyl-ACP thioesterase. Only one acyl-ACP thioesterase has
11 been identified to date in *P. tricornutum* (PtTE, Phatr3_J33198) but it does not share sequence
12 similarities with plant or bacterial thioesterases. Overexpression of PtTE in *P. tricornutum*
13 does not alter the profile of total fatty acids but causes a 72% increase in total lipids [47].
14 Heterologous expression of a lauric acid biased thioesterase (C12-TE) from *Umbellularia*
15 *California* and a myristic acid biased thioesterase (C14-TE) from *Cinnamomum camphora* in
16 *P. tricornutum* led to an increase in short chain fatty acid synthesis in *P. tricornutum* [48].
17 The same kind of result has been obtained in several species of green microalgae [49-51].

18
19 In the cytosol, FFAs need to be thioesterified to a coenzyme A by an acyl-CoA synthase
20 (ACS) to be used as substrates for the eukaryotic pathway. This export outside the chloroplast
21 is a major subject of study because it is almost unknown in diatoms. Eventually, VLC-PUFAs
22 are imported back to the plastid to serve as precursors for glycerolipid syntheses. The VLC-
23 PUFA import mechanism remains to be established (see below) and has been called the
24 "omega" pathway [13, 26] (see 2.3).

25 2.2. Synthesis of glycolipids in the secondary plastids

26 From cyanobacteria to secondary endosymbionts, the glycerolipid composition in the
27 chloroplast is remarkably conserved. It consists of a quartet of four lipid classes, *i.e.*
28 monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG),
29 sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) [52] (Figure 1). These
30 four lipid classes are conserved in diatoms. Nevertheless, the precise composition of each of
31 the four membranes is still unknown [5, 26].

32 The "prokaryotic" pathway classically begins with the esterification of fatty acid originating
33 from an acyl-ACP at the *sn*-1 position of the glycerol-3-phosphate backbone by a glycerol-3-
34 phosphate acyltransferase 1 (ATS1) forming lysophosphatidic acid (LPA). A second acyl
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1 group is esterified, at the *sn*-2 position, by 1-acylglycerol-3-phosphate-acyltransferase (ATS2)
2 forming phosphatidic acid (PA) [53-55]. Respectively, one and two homologs of ATS1
3 (Phatr3_J3262) and ATS2 (ATS2a, Phatr3_J11916 and ATS2b, Phatr3_J43099) were
4 identified in *P. tricornutum* [5]. PA can be used for i) the synthesis of CDP-DAG by a plastid
5 CDP-DAG synthase (CDP-DAGS, Phatr3_J7678 in *P. tricornutum*), which substitutes the
6 polar head of PA by a cytidine diphosphate (CDP). CDP-DAG can then be converted into PG
7 by a phosphatidylglycerol phosphate synthase (PGPS, Phatr3_J8663). Alternatively, PA can
8 be utilized to synthesize diacylglycerol (DAG) by the action of a plastid phosphatidate
9 phosphatase (PAP) (for review, [26, 52]).

16 DAG is then the precursor of the synthesis of SQDG and MGDG. The polar head of SQDG is
17 classically formed from UDP-glucose by condensation with a molecule of sulfite (SO_3^{2-}) by a
18 UDP-sulfoquinovose synthase (SQD1, Phatr3_J21201 in *P. tricornutum*) [56]. Transfer of
19 sulfoquinovose to DAG is then achieved by a UDP-sulfoquinovose:DAG
20 sulfoquinovosyltransferase (SQD2) forming SQDG [57]. Four genes code for putative UDP
21 sulfoquinovose: DAG sulfoquinovosyltransferases in *P. tricornutum* (SQD2, Phatr3_J50356;
22 SQD2-Like-A, Phatr3_EG02454; SQD2-Like-B, Phatr3_J54068 and SQD2-Like-C,
23 Phatr3_J22554).

31 MGDG synthesis is catalyzed by MGDG synthases (MGDs). These enzymes transfer
32 galactose from uridine diphosphogalactose (UDP-Gal) to DAG [58-60]. The formation of
33 DGDG is catalyzed by DGDG synthases (DGDs), adding a second galactose from a UDP-Gal
34 molecule. Homologues for all these enzymes can be detected in the genome of *P.*
35 *tricornutum*, *T. pseudonana*, *Fistulifera*, and other diatoms [5] and a clear future challenge
36 will be to establish their precise localization and roles in diatoms. Due to the predominance of
37 photosynthetic organisms on Earth, MGDG and DGDG are the two most abundant lipids in
38 the biosphere indispensable for the operation of the photosynthetic machinery (for review,
39 [52]). MGDG and DGDG are probably main components of thylakoids in secondary plastids,
40 possibly present in some of the plastid limiting membranes, but their precise localization
41 remains to be demonstrated [26, 61].

52 2.3. Synthesis of phospholipids and betaine lipids in the endoplasmic reticulum

53 The ER is the second important site for glycerolipid metabolism. The ER is part of the
54 endomembrane system (ER, Golgi, trans-Golgi network, nuclear envelope lysosomes,
55 vacuoles, vesicular trafficking network, plasma membrane, etc.) and is connected therefore
56 with the outermost membrane of the secondary plastid [9, 28] (Figure 1). The ER is the site
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1 of elongation of fatty acids leading to chain lengths greater than 20 carbons [62]. The
2 production of VLC-PUFAs such as eicosapentaenoic acid (EPA, 20:5) involves elongases
3 (ELO) adding two carbons to the aliphatic chain, but also desaturases (FADs) adding double
4 bonds [42-44]. Desaturation of fatty acids takes place both within chloroplast and in the ER.
5 A number of desaturases have been identified in *P. tricornutum* by sequence homology with
6 other organisms [42] and an update of the fatty acid desaturation pathways in diatoms has
7 recently been performed [13]. Several desaturases (PtD5p, PtD6p, PtFAD2, PtFAD6) have
8 been functionally characterized in *P. tricornutum* by heterologous expression in
9 *Saccharomyces cerevisiae* and *Synechococcus* [63, 64]. Overexpression of a delta 5
10 desaturase (called PtD5b) in *P. tricornutum* led to an increase in PUFAs synthesis [65].
11 Similarly, the heterologous expression of delta 5 elongase (OtElo5) and delta 6 desaturase
12 (OtD6) from the green picoalga *Ostreococcus tauri* in *P. tricornutum* led to an increase in
13 DHA synthesis in TAGs [66].

23 The ER is also the site of synthesis of membrane glycerolipids, including
24 phosphoglycerolipids or phospholipids (Figure 1, PL). Phosphatidic acid (PA) and DAG are
25 at the origin of all PLs. PA is the precursor of phosphatidylglycerol (PG), phosphatidylinositol
26 (PI) and phosphatidylserine (PS) especially through a cytosolic CDP-DAG pathway, whereas
27 DAG is a precursor of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) through
28 the Kennedy pathway. PC is the most abundant phospholipid in diatoms, and possibly mainly
29 present in the endoplasmic reticulum [5].

36 Betaine lipids (Figure 1, BL) are a class of glycerolipids occurring naturally in lower
37 eukaryotic organisms such as algae, bryophytes, fungi and some primitive protozoa, and in
38 some photosynthetic bacteria [67]. The three main BL detected in microalgae are 1,2-
39 diacylglyceryl-3-O-2'-(hydroxymethyl)-(N,N,N-trimethyl)- β -alanine (DGTA), 2-
40 diacylglyceryl-3-O-4'-(N,N,N-trimethyl)-homoserine (DGTS) and 1,2-diacylglyceryl-3-O-
41 carboxy-(hydroxymethyl)-choline (DGCC). BL have two fatty acids esterified at the *sn*-1 and
42 *sn*-2 positions, as well as a betaine polar group linked by an ether bond at the *sn*-3 position.
43 Knowledge about BL in microalgae is still scarce. The synthetic route of DGTS was mainly
44 studied in *Chlamydomonas reinhardtii*, a primary endosymbiont and in *Nannochloropsis*
45 *oceanica*, a secondary endosymbiont [68]. Synthesis of DGTS is carried out by a betaine lipid
46 synthase (BTA1) [69]. The synthesis of DGTA is still elusive, as multiple enzymatic activities
47 might be involved. It is currently considered that synthesis of DGTA in *P. tricornutum* could
48 be achieved by a betaine-lipid synthase-like (BTA-like – Phatr3_J42872) enzyme [6, 7, 68]. A
49 phosphate deficiency is responsible for an accumulation of BL in compensation of the
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1 degradation of PLs [5, 70]. Specific molecular species of DGTA have been detected at the
2 surface of lipid droplets purified from *P. tricornutum* deficient in nitrogen [9].

3 Productions of VLC-PUFAs and of glycerolipids are tightly connected. In the pennate diatom
4 *P. tricornutum*, both PC and DGTA are thus considered as platforms for the synthesis of EPA
5 [5]. EPA is then directed to the plastid for the synthesis of MGDG, DGDG, SQDG and PG.
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7 Our knowledge on the machinery involved in this import, called the “omega” pathway [13,
8 26], is very poor and its dissection represents one of the most important challenges for the
9 future.
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14 15 **2.4. Where is synthesized triacylglycerol?**

16 TAGs are highly hydrophobic glycerolipids accumulating in the form of lipid droplets in the
17 cytosol of *P. tricornutum* in case of nutritional starvation and abiotic stress [5-7, 9, 10, 71].
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19 Two different pathways can carry out the synthesis of TAGs in the ER or ER-related
20 compartment: i) an acyl-CoA dependent pathway commonly referred to as the Kennedy
21 pathway (**Figure 2**) and ii) an acyl-CoA independent pathway involving phosphatidylcholine
22 (PC) as an acyl donor.
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29 The Kennedy pathway corresponds to a *de novo* synthesis of TAGs from fatty acids derived
30 from the cytosolic pool of acyl-CoA. The route begins with an esterification of the glycerol-3-
31 phosphate backbone with an acyl-CoA by a glycerol-*sn*-3-phosphate acyltransferase (GPAT)
32 forming lyso-phosphatidic acid (LPA). Overexpression of GPAT in *P. tricornutum*
33 (Phatr3_J54709) causes an increase in the amount of neutral lipids (determined by Nile Red
34 staining) by a factor 2 as well as a higher proportion of unsaturated fatty acids [72]. LPA is
35 esterified with a second acyl-CoA by a lysophosphatidate acyltransferase (LPAAT) forming
36 phosphatidic acid (PA). One LPAAT called AGPAT1 (Phatr3_J45551), presenting four
37 conserved acyltransferase motifs I-IV, has been identified in *P. tricornutum* [73].
38 Overexpression of AGPAT1 is responsible for an increase in TAGs and a decrease in the
39 content of carbohydrates and soluble proteins. PA is then dephosphorylated by a phosphatidic
40 acid phosphatase (PAP) producing diacylglycerol (DAG). Two putative PAP genes were
41 identified in *P. tricornutum* [46]. An acyl-CoA can also serve as substrate for a diacylglycerol
42 acyltransferase (DGAT) esterifying a third fatty acid to DAG, leading to the formation of
43 TAG (**Figure 2**). Unlike higher plants where three types of DGAT were discovered (called
44 DGAT1, DGAT2 and DGAT3) [74], only two types of DGAT were described in diatoms
45 (DGAT1 and DGAT2) [46, 75-77]. The genome of *P. tricornutum* codes for 1 isoform of
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1 DGAT1 (PtDGAT1, Phatr3_J9794) and 4 isoforms of DGAT2 (PtDGAT2A, Phatr3_J49462;
2 PtDGAT2B, Phatr3_J49544; PtDGAT2C, Phatr3_J31662 and PtDGAT2D, Phatr3_J43469).
3 PtDGAT1 contains at least 8 transmembrane domains [75] and isoforms of PtDGAT2 possess
4 1 to 4 transmembrane domains [46, 77]. Overexpression of PtDGAT2A in *P. tricornutum*
5 causes a 34.8% increase in the amount of neutral lipids [76]. Likewise, a second study showed
6 that the overexpression of PtDGAT2D led to a 1.4-fold and 2-fold increase in the amount of
7 TAGs and total lipid content, respectively [78]. The authors also showed by dynamic
8 incorporation of ¹³C-labeled bicarbonate that the overexpressing lines exhibited an increase in
9 carbon flux towards pyruvate and acetyl-CoA that may explain the increase in the amount of
10 lipids observed [78].
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20 In higher plants and yeast, alternative routes to the Kennedy pathway can involve PC as an
21 acyl donor [79, 80]. For instance, a PC:acyltransferase (PDAT) can transfer a fatty acid from
22 the *sn*-2 position of PC to the *sn*-3 position of DAG, thus forming a TAG molecule and Lyso-
23 PC as a co-product [81]. There is no demonstration of the existence of this pathway in
24 Heterokonta but a gene encoding a putative PDAT (Phatr3_J8860) has been detected in the
25 genome of *P. tricornutum*, and has been proposed to also act in TAG biosynthesis [42, 82].
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31 The recent purification of the lipid droplet from *P. tricornutum* cells starved in nitrogen has
32 highlighted the presence of PC, SQDG and specific molecular species of DGTA in the lipid
33 droplet glycerolipidome [9]. The authors propose that these three glycerolipids are
34 components of the polar lipid monolayer of the lipid droplet. By comparison with other
35 eukaryotes, PC and DGTA could be markers of the ER. However, the presence of SQDG,
36 classically synthesized in plastid membranes, is puzzling and may suggest a connection
37 between the lipid droplet and the plastid. Since the outermost membrane of the plastid is
38 connected with the endomembrane system, one could speculate that it is a site of production
39 of TAG, in the specific condition of nitrogen starvation. Other site(s) might be involved in
40 other stress conditions.
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50 3. Overview of the metabolism of steroids in diatoms

51 Steroids are essential building blocks for eukaryotic organisms especially for the curvature
52 and the flexibility of the membranes [83]. Diatom sterol metabolism was initially
53 reconstructed *in silico* using the DiatomCyc v2.0 model, based on genome annotations and
54 validations by functional genomics [84, 85]. The synthesis of sterols could theoretically be
55 fueled by two independent metabolic pathways, *i.e.* the mevalonate (MVA) pathway and the
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1 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway,
2 also known as the non-mevalonate pathway (**Figure 1** and **Figure 2**). In diatoms the MVA
3 pathway localized in the cytosol seems the major route for the synthesis of sterols, while the
4 MEP pathway, located in the plastid, appears to preferentially feed the synthesis of
5 carotenoids [85-88]. These two routes allow the synthesis of the precursors required for the
6 synthesis of sterols, the five-carbon building blocks isopentenyl-pyrophosphate (IPP) and its
7 isomer dimethylallyl-pyrophosphate (DMAPP) [85, 87, 88] (**Figure 1** and **Figure 2**).

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13 The MVA pathway operates in six steps initiated by the condensation of two molecules of
14 acetyl-CoA into acetoacetyl-CoA by acetyl-CoA C-acetyltransferase (ACAT) [85]. A third
15 acetyl-CoA molecule is condensed to form acetoacetyl-CoA by the action of a
16 hydroxymethylglutaryl-CoA synthase (HMGS) forming S-3-hydroxy-3-methylglutaryl-CoA
17 (HMG-CoA). Then, HMG-CoA is reduced by an HMG-CoA reductase (HMGR) into
18 mevalonate. HMGR is localized in the endoplasmic reticulum in a large number of model
19 organisms [89-91], however the precise localization of this enzyme is not known in diatoms.
20 Finally, mevalonate is converted in three steps into IPP, *i.e.* two successive phosphorylation
21 reactions catalyzed by a mevalonate kinase (MK) and a phosphomevalonate kinase (PMK),
22 forming mevalonate-P and mevalonate-pyrophosphate, and an ATP elimination reaction
23 catalyzed by a diphosphomevalonate decarboxylase (MVD) [87].

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34 DMAPP and IPP are condensed, forming geranyl-pyrophosphate (GPP). The final addition of
35 IPP to GPP generates farnesyl-pyrophosphate (FPP). FPP is converted by a squalene synthase
36 (SQS) in presqualene-PP then into squalene [85]. In *P. tricornutum*, the sequences of SQS
37 and Isopentenyl-diphosphate δ -isomerase (IDI) are fused [85, 88]. However, the *P.*
38 *tricornutum* genome encodes an independent second IDI (Phatr3_J12533) [85]. Whatever the
39 organism studied, the 30-carbon squalene is the precursor of all steroids. Squalene is oxidized
40 by a squalene epoxydase (SQE) into 2,3-oxidosqualene. However, this enzyme was not
41 detected in the genomes of the pennate diatom *Phaeodactylum tricornutum* or the centric
42 diatom *Thalassiosira pseudonana* [85, 92]. It was suggested that an alternative SQE may exist
43 in *P. tricornutum* based on pharmacological analyzes showing the no inhibitory effect of
44 terbinafine, an SQE inhibitor [85]. The existence of this alternative *P. tricornutum* SQE
45 (AltSQE – Phatr3_J45494) has recently been demonstrated [83]. Unlike SQE which is a
46 flavoprotein, AltSQE is a fatty acid hydroxylase, localized in the ER using a fusion with a
47 mVenus yellow fluorescent protein (YFP) protein, coinciding therefore with the location of
48 SQE classically observed in eukaryotes [83, 93, 94]. Nevertheless, no difference could be
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1 observed in sterol accumulation in *AltSQE* overexpressors or corresponding RNA interference
2 (RNAi) lines in *P. tricornutum* [83]. Subsequently, 2,3-oxidosqualene is cyclized into
3 cycloartenol by an oxidosqualene cyclase (OSC), which is a cycloartenol synthase (CAS)
4 [85]. Our understanding of sterol metabolism in diatoms becomes fragmentary from this
5 point. In *P. tricornutum*, twenty steps are necessary to reach the final product of sterol
6 synthesis, brassicasterol [85]. Both end products, campesterol (24-methylcholest-5-en-3 β -ol)
7 and brassicasterol (24-methylcholest-5,22-dien-3 β -ol) were detected by GC-MS [85, 95].
8 Conversion of campesterol into brassicasterol was confirmed by the use of imidazole, an
9 inhibitor of cytochrome P450 proteins of the CYP70 subfamily [85]. Recently, the
10 architecture (*i.e.* proteome, glycerolipidome, pigment and sterol composition) of the lipid
11 droplet of *P. tricornutum* has been determined [9]. Only the presence of free brassicasterol
12 (<5 mol% compared to sterol + glycerolipids) could be detected in the purified lipid droplets.
13 Based on the polarity of brassicasterol and its proportion detected in the purified fraction of
14 lipid droplets, a localization of brassicasterol at the level of the monolayer of polar lipids was
15 proposed [9].
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27 **4. The impairment of sterol metabolism triggers an accumulation of TAGs**

28 Understanding a metabolic pathway can be achieved through chemical engineering
29 approaches, by the use of specific inhibitors of a particular pathway enzyme, in complement
30 with genetic analyses of appropriate mutants. In the initial study of sterol synthesis [85], an
31 intriguing question was the absence of a canonical SQE, raising the question on the way *P.*
32 *tricornutum* used 2,3 epoxysqualene for sterol synthesis. To address this question, two
33 chemical inhibitors were used, *i.e.* terbinafine, inhibiting SQEs, and Ro 48-8071, inhibiting
34 OSCs. *P. tricornutum* cells did not appear to show any growth arrest after treatment with 40
35 μ M terbinafine, whereas treatment with 30 μ M Ro 48-8071 killed cells in 48 hours. The
36 authors analyzed the sterol composition following the use of these inhibitors. A treatment
37 with 10 μ M of Ro 48-8071 was responsible for an accumulation of squalene and 2,3
38 epoxysqualene consistently with the cyclization of 2,3 epoxysqualene into squalene by an
39 OSC in *P. tricornutum* (called PtOSC in this article). In addition, after Nile red staining, lipid
40 droplets were observed in the cells treated with the OSC inhibitor [7, 85].
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54 A second approach to dissect the steroid synthesis pathway in diatoms is the use of RNAi to
55 silence the expression of target genes at the transcriptional level. RNAi corresponding to an
56 oxidosqualene cyclase gene were generated, showing a decrease in cell growth coupled with a
57 decrease in sterol synthesis and a high accumulation of lipid droplets [85]. Together, these
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chemical and genetic analyses suggested that an alteration of the sterol synthesis pathway could lead to a relocation of carbon in *P. tricornutum* from sterols to TAGs.

Independently, a screening of small molecules was set up in the diatom *P. tricornutum* in order to discover biological processes that may be involved in the synthesis of TAG [7]. This chemical screening was performed using the Prestwick library of 1.280 compounds, for which biological targets had been previously identified in human or other organisms. This study was carried out with the underlying hypothesis of mimicry or structural relationship between obtained hit compounds and biomolecules involved in the *in vivo* signaling process, or metabolites. Among validated hit compounds, five molecules known to interact with the mevalonate pathway and sterol metabolism were identified: mevastatin and simvastatin, known to target HMGRs [96-98], ketoconazole targeting a sterol-14- α -demethylases [99], and eventually estrone and ethynylestradiol. The detection of a large number of inhibitors of the sterol pathway, together with whole genome expression analyses of **treated** cells, strongly suggested that there was a likely competition for carbon at the level of acetyl-CoA between sterol and TAG syntheses [7].

In parallel, it has also been shown that the inhibition of Target of Rapamycin (TOR) kinase by 2 μ M of AZD-8055 in *P. tricornutum* affects the cell concentration and promotes the accumulation of TAG in the form of lipid droplets after different kinds of staining [100, 101]. The same type of response has been observed in the green algae *Chlamydomonas reinhardtii* [102]. A decrease in brassicasterol level in *P. tricornutum* following this treatment has also been observed, suggesting **a convergence of TOR-mediated signaling and energy metabolism (lipid and sterol) in *P. tricornutum*** [100].

5. The question of the effect of synthetic analogs of sterols on diatoms: case study of ethynylestradiol

Following the screening of small molecules in *P. tricornutum*, the effect of ethynylestradiol was further investigated [7]. This compound is a synthetic derivative of the natural hormones estrone and estradiol [103] used in birth control pills [104]. These molecules are well-known endocrine disruptors, with recorded utilization in aquaculture for the development of single-sex populations of fish [105]. The mode of action of ethynylestradiol in *P. tricornutum* is currently unknown but this compound may target cytochrome P450 (CYP) proteins [7, 106]. Following treatment an increase in Nile Red staining coupled with a decrease in *P. tricornutum* cell growth was observed (for concentrations $>8 \mu$ M ethynylestradiol) [7]. A

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small effect on the activity of photosystem II was observed (>30 μM ethynylestradiol). The glycerolipid profile of cells treated 48 hours in absence or presence of 30 μM ethynylestradiol highlighted a strong accumulation of TAG, confirming the Nile Red staining data, consistent with an accumulation of TAG following a modification of the sterol synthesis pathway. The same kind of response was observed with 15 μM simvastatin, an inhibitor of HMGR. For all glycerolipids, with the exception of SQDG, a decrease in the proportion of EPA was observed following treatment. In addition, PC and DGTA were enriched in C18 precursors of EPA (18:1 to 18:3). As the synthesis of EPA is a very fast mechanism, the presence of these intermediates suggests that treatment with ethynylestradiol could disrupt a metabolon involved in VLC-PUFAs production.

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A transcriptomic analysis of *P. tricornutum* treated with 0, 10 and 20 μM of ethynylestradiol was performed [7]. Expression of genes involved in the MEP/DOXP pathway was not impaired by treatment with ethynylestradiol. On the other hand, important alterations in the mevalonate pathway were noted, with increased expression of genes coding for a HMGS, a geranyl-diphosphate/farnesyl-diphosphate synthase, and a sharp decreased expression of genes coding for a MK and a putative geranyl-diphosphate synthase. Finally, an increase in the expression of genes putatively coding for enzymes of the sterol synthesis pathway, in particular cytochrome P450 (CYP) **enzymes** was detected following treatment with ethynylestradiol. At the glycerolipid metabolism level, some genes coding for TAG catabolism were downregulated whereas genes coding for the biosynthesis of acetyl-CoA were upregulated. The lipidome and transcriptome analyses performed in parallel were therefore consistent with a relocation of carbon from sterols to TAGs. Together these results suggested that ethynylestradiol might target specific CYP, which role needs to be determined in the future. An increase in gene expression of the VLC-PUFAs pathway was induced by ethynylestradiol, with the noticeable exception of a gene encoding $\Delta 6$ -ELO-B1, coinciding with the interruption of the VLC-PUFAs pathway observed at the level of PC and DGTA. A more in-depth investigation of this $\Delta 6$ -ELO-B1 may allow in the future to understanding the effects of ethynylestradiol in the VLC-PUFAs pathway.

51 52 53 **6. Conclusion and future lines of research**

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Although knowledge of metabolic pathways deciphered in yeast, *Arabidopsis* or *Chlamydomonas* is extremely useful to mine the genome of diatoms and propose metabolic schemes, this mini-review highlight gaps that need to be filled, which cannot benefit from comparative analyses with previous models. Most importantly, the understanding of

1 metabolic processes occurring in the secondary plastid (**Figure 1**) cannot be addressed simply
2 based on knowledge from plant and algae (algae = green algae, red algae and Glaucophytes)
3 as the secondary plastid differs strongly in its structure and function with the primary
4 chloroplast [26, 107, 108]. This question is critical when addressing the fatty acid and sterol
5 metabolic networks. The proteomic and lipidomic compositions of the four limiting
6 membranes of diatom's plastid are unknown; the connection between the two outermost
7 membranes and the nuclear envelope is a real puzzle. The lipid droplet from *P. tricornutum*
8 cells, starved in nitrogen, is the only purified subcellular compartment analyzed to date,
9 highlighting a possible connection with the outermost membrane of the plastid. Future
10 challenges include therefore the characterization of the proteomes of compartments that are
11 specific to diatoms. In some instances, like the synthesis of VLC-PUFAs and its allocation to
12 specific glycerolipid classes, the absence of metabolic intermediates between the initial
13 substrate and the end products suggests a possible channeling *via* large-size complexes or
14 structures, called "metabolons" [109, 110]. Metabolic modules can be pointed in a diatom
15 cell: (1) fatty acids are synthesized in the stroma of the plastid; (2) fatty acid elongation and
16 desaturation occur mainly on PC and DGTA, acting therefore like "lipid platforms" for VLC-
17 PUFAs production (in a domain of the endomembrane system that needs to be identified); (3)
18 the synthesis of VLC-PUFAs is tightly coupled with their import to the core of the plastid *via*
19 the so-called "omega" pathway; (4) sterols are mainly synthesized using precursors deriving
20 from the mevalonate pathway. These metabolic modules can be characterized functionally by
21 genetic analyzes or chemical treatments with appropriate inhibitors. At the junction of these
22 modules, metabolic intermediates can therefore play dramatic roles in directing carbon fluxes
23 from one direction to another. Here, acetyl-CoA seems determinant in the balance between
24 TAGs and sterols, although the mass ratio between these two carbon sinks are different. The
25 impairment of sterol metabolism is not only compensated by an increase in TAG, as
26 illustrated by the disruption of the VLC-PUFAs pathway and because a structural and
27 functional impact on membranes is likely to occur following a sterol decrease. Acetyl-CoA
28 might therefore not act simply as a limiting factor. Future studies will also need to assess the
29 dynamics of the relationship between acyl-lipid and sterol metabolism. It will be important to
30 determine if any physiological regulation of acetyl-CoA homeostasis is involved in the
31 control of the acyl-lipid / sterol balance. Thus, the metabolic connections summarized in this
32 review are valuable to propose strategies to control the level in TAG in microalgae for
33 multiple biotechnological applications, but they also highlight important challenges to
34 advance our understanding of lipid metabolism in secondary endosymbionts.

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Figure Legends

Figure 1: Hypothetical scenario of synthesis pathways of sterols and glycerolipids in the pennate diatom *Phaeodactylum tricornutum*. Cell compartments: cERM, chloroplast endoplasmic reticulum membrane (continuous with the outer nuclear envelope membrane); pPM, periplastidial membrane; oEM and iEM, outer and inner envelope membrane, respectively; ppc, periplastidial compartment. Glycerolipid metabolism: FAS, fatty acid synthase; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PL, phospholipid; BL, betaine lipid; TAG, triacylglycerol. Sterol metabolism: AltSQE, alternative squalene epoxydase; MEP/DOXP, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate; MVA, mevalonate; IPP, isopentenyl-pyrophosphate; DMAPP, dimethylallyl-pyrophosphate.

Figure 2: Competing sinks for carbon in diatoms. The red dashed arrows indicate several steps in the sterol synthesis pathway. Abbreviations in alphabetical order – Acetyl-CoA and Pyruvate precursors: ACL, acetyl-CoA lyase; ACS, acetyl-CoA synthetase; ME, Malic enzyme; PDH, pyruvate deshydrogenase; PEP, phosphoenolpyruvate; PFL, pyruvate formate lyase; PK, Pyruvate kinase. Glycerolipid metabolism: ACCase, acetyl-CoA carboxylase; DGAT, diacylglycerol O-acyltransferase; ER, enoyl-ACP reductase; GPAT, glycerol-*sn*-3-phosphate acyltransferase; HAD, hydroxyacyl-ACP-reductase; KAR, 3-ketoacyl-ACP-reductase, NADPH-dependent; KASI, ketoacyl-ACP synthase I; KASII, ketoacyl-ACP synthase II; KASIII, β -ketoacyl-ACP synthase III; LPAAT, 1-acylglycerol-3-phosphate O-acyltransferase; MCAT, malonyl-CoA:acyl-carrier protein transacylase; PAP, phosphatidate phosphatase. Sterol metabolism: ACAT, acetyl-CoA C-acyltransferase; AltSQE, alternative squalene epoxydase; CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP, 4-

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diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol; CMS: 2-C-methyl-D-erythritol 4-phosphate; DMAPP, dimethylallyl-pyrophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FPP, farnesyl-pyrophosphate; G3P, glyceraldehyde-3-phosphate; GPP, geranyl-pyrophosphate; HDR, (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate reductase; HDS, (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate synthase; HMB-PP, (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate; HMG-CoA, S-3-hydroxy-3-methylglutaryl-CoA ; HMGR, HMG-CoA reductase; HMGS, hydroxymethylglutaryl-CoA synthase; IPP, isopentenyl-pyrophosphate; MCS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-C-methylerythritol 4-phosphate; MEP/DOXP, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate; MK, mevalonate kinase; MVA, mevalonate; MVD, diphosphomevalonate decarboxylase; OSC, oxidosqualene cyclase; PMK, phosphomevalonate kinase; SQS, squalene synthase.

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secondary plastid (4 limiting membranes)

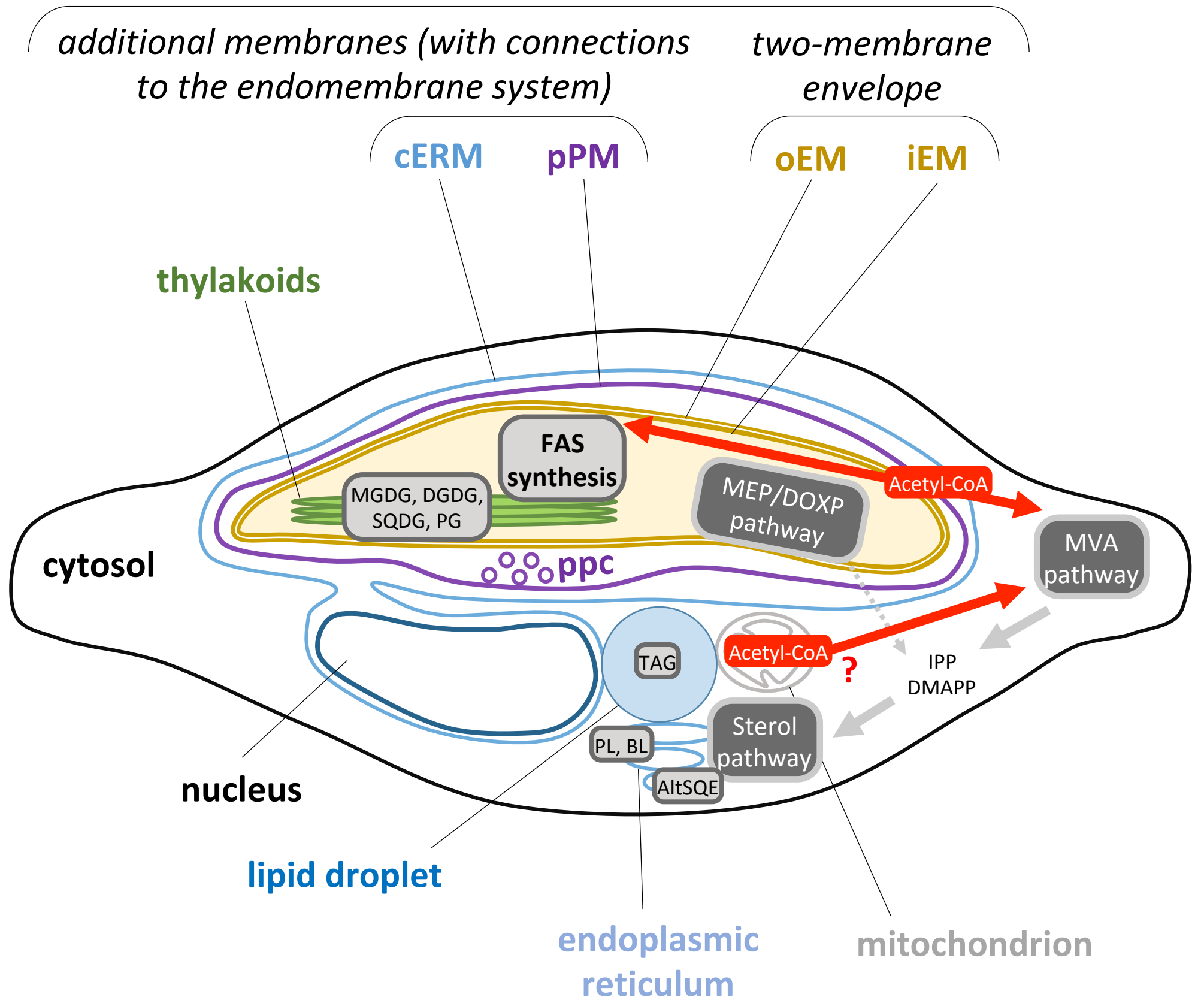


Figure 2 - Maréchal & Lupette

