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Title Page

Genome analysis of sphingolipid metabolism-related genes in *Tetrahymena thermophila* and identification of a Fatty Acid 2 Hydroxylase involved in the sexual stage of conjugation

Running Title:

Sphingolipid genes in *Tetrahymena thermophila*

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Tetrahymena, sphingolipid, hydroxy fatty acids, conjugation, ciliates, fatty acid 2-hydroxylase

Summary
Sphingolipids are bioactive lipids present in all eukaryotes. *Tetrahymena thermophila* is a ciliate that displays remarkable sphingolipid moieties, i.e., the unusual phosphonate-linked headgroup ceramides, present in membranes. To date, no identification has been made in this organism of the functions or related genes implicated in sphingolipid metabolism. By gathering information from the *T. thermophila* genome database together with sphingolipid moieties and enzymatic activities reported in other *Tetrahymena* species, we were able to reconstruct the putative *de novo* sphingolipid metabolic pathway in *T. thermophila.* Orthologous genes of 11 enzymatic steps involved in the biosynthesis and degradation pathways were retrieved. No genes related to glycosphingolipid or phosphonosphingolipid headgroup transfer were found, suggesting that both conserved and innovative mechanisms are used in ciliate. The knockout of gene TTHERM_00463850 allowed to identify the gene encoding a putative fatty acid 2-hydroxylase, which is involved in the biosynthesis pathway. Knockout cells have shown several impairments in the sexual stage of conjugation since different mating types of knockout strains failed to form cell pairs and complete the conjugation process. This

fatty acid 2-hydroxylase gene is the first gene of a sphingolipid metabolic pathway to be identified in ciliates and have a critical role in their sexual stage.

Abbreviations

C1P: Ceramide-1-phosphate

CAEP: ceramide 2-aminoethylphosphonate

Cer: ceramide

CMAEP: ceramide N-methyl-2-aminoethylphosphonate

EPC: ethanolamine-phosphorylceramide synthase

FA2H: fatty acid 2 hydroxylase protein

FA2H: fatty acid 2 hydroxylase gene

FA2H-EGFP: fatty acid 2 hydroxylase-EGFP fusion strain

FAH: fatty acid hydroxylase

IPC: inositol-phosphorylceramide synthase

KOC5DES : C-5 sterol desaturase mutant strain

KOFA2H: fatty acid 2 hydroxylase knockout mutant strain

S1P: sphingosine-1-phosphate

SMP: sphingolipid metabolic pathway

SMS1/2: sphingomyelin synthases ½

Sph: sphingosine

SPT: serine palmitoyltransferase

Introduction

The identification and analysis of bioactive lipids have received significant attention in the past 25 years, and the discoveries of the key roles played by lipids now assure that this field will increasingly be in the spotlight of cell biology investigation over the course of this century. Single lipid species involved in energy metabolism, membrane structure, and signaling are among the key breakthroughs. For instance, a pioneering work showed that protein kinase C (PKC) and calcium release are regulated by diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) (Nishizuka 1992). Eicosanoids, which are involved in inflammatory responses, constitute another example of lipids acting as signaling molecules (Serhan & Savill 2005). Other studies have reported that phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3), phosphatidic acid (PA), monoacylglycerols and anandamide (ligands for cannabinoid receptors), lyso-phosphatidic acid and platelet activating factor (PAF) have proved to act as regulatory molecules or be implicated in cell signaling.

Sphingolipids are a class of bioactive lipids of special interest. This complex class of lipids has a common sphingosine backbone resulting from the *de novo* synthesis of serine and a long-chain fatty acyl-CoA, later converted into ceramides, phosphosphingolipids, glycosphingolipids, as well as a wide range of additional species (Hannun & Obeid 2008). Sphingolipids are found in eukaryotic organisms and in some anaerobic bacteria such as *Fusobacterium, Sphingomonas, Sphingobacterium* and others (Olsen & Jantzen 2001).

Sphingolipids can be divided into the following classes: sphingoid bases (long-chain bases) and their simple metabolites (such as sphingoid base/ 1-phosphate), sphingoid bases with an amide-linked fatty acid (e.g., ceramides), and more complex sphingolipids with head groups that are linked by phosphodiester bonds (phosphosphingolipids), glycosidic linkages (simple and complex glycosphingolipids such as cerebrosides and gangliosides), and other groups (such as phosphono- and arseno-sphingolipids). Although sphingolipids are diverse in structure and function, they all share synthetic and catabolic pathways in their synthesis and turnover. Thus, sphingolipid metabolism can be described as a number of interrelated systems emerging from a single common entry point and converging into a single common breakdown pathway (Gault, Obeid & Hannun 2010).

Several bioactive molecules that are members of the sphingolipid family, including ceramide (Cer), sphingosine (Sph), Sph-1-phosphate (S1P), and Cer-1-phosphate (C1P), play different roles such as signal transduction, protein targeting regulation and intracellular membrane trafficking, mediation of cell-cell interactions and recognition, thus contributing to many cellular events (Hannun & Obeid 2017). The intensification of studies in the past years is showing other bioactive sphingolipid moieties such as 2-hydroxy ceramides (Kota & Hama 2014). This species is synthesized by the fatty acid 2-hydroxylase, introducing a hydroxyl group during the de novo ceramide synthesis. Demyelination of both central and peripheral nervous systems is frequently associated with its alteration in humans and mice, which generally leads to the onset of neurodegenerative diseases (Edvardson et al. 2008; Potter et al. 2011). The enzyme has also shown to regulate the differentiation of various cell types including adipocytes, keratinocytes, and megakaryocytes (Uchida et al. 2007; Guo et al. 2010; Jin et al. 2016).

Historically, a variety of model systems have contributed to research on sphingolipids. These include both mammalian and invertebrate cells as well as fungi such as budding yeast (Acharya & Acharya 2005; Merrill 2011; Montefusco, Matmati & Hannun 2014). One group of promising organisms that are just beginning to be tapped are the ciliates and in particular the experimentally versatile species *Tetrahymena thermophila*, a free-living unicellular ciliated protozoan that is widely found in freshwater habitats. Despite being a unicellular organism, *Tetrahymena* shares a wide number of cell structures and molecular processes that are also observed in multicellular eukaryotic organisms. In particular, many human proteins share orthologs with *Tetrahymena* that are not found in other unicellular models such as yeast (Eisen et al. 2006). Tools for sophisticated genetic manipulation, e.g., gene knockout by homologous recombination, simultaneous deletion of multiple genes within a gene family, gene tagging and overexpression, are well established (Ruehle, Orias & Pearson 2016). Indeed, *Tetrahymena* has been a widely used eukaryotic model organism in biological research. Many advances have emerged in molecular biology in this eukaryotic organism, especially the elucidation of the characteristics of telomers (Blackburn & Gall 1978) and telomerase (Greider & Blackburn 1985), the self-splicing RNA process (Kruger et al. 1982), the first revelation that a transcription factor was a histone modifying enzyme (Taverna, Coyne & Allis 2002) and one of the initial descriptions of the role played by small RNAs in the formation of heterochromatin (Mochizuki et al. 2002; Mochizuki &

Gorovsky 2004). *Tetrahymena* is also widely exploited at the biotech level (Elguero, Nudel & Nusblat 2018).

Over more than fifty years *Tetrahymena* has been used in many classical studies on lipid metabolism and membranes (Taketomi 1961; Thompson 1967). Most recently, several groups have focused on phosphatidylinositol phosphates (Kurczy et al. 2010; Leondaritis et al. 2011), and polycyclic triterpenoids such as hopanoids and sterols (Tomazic et al. 2014). In addition, there have been important works on membrane dynamics such as membrane junctions, membrane curvature and traffic in the endomembrane system, which depend on lipid determinants (Nusblat, Bright & Turkewitz 2012).

Nevertheless, there has been only very limited work on sphingolipids in ciliates, chiefly concerned with the identification of a number of moieties present in subcellular fractions (Viswanathan & Rosenberg 1973; Kaya, Ramesha & Thompson 1984a; Kaneshiro 1987) and the identification of some enzymatic activities. Preliminary studies in Paramecium suggested that sphingolipids and phosphonolipids would play a critical role in controlling the ion channels involved in the locomotor capacity of this ciliate, based on the evidence that a barium-sensitive locomotory mutant of *P. tetraurelia,* baA, carried altered sphingolipid and phosphonolipid compositions (Forte et al. 1981).

To date, no related gene implicated in sphingolipid metabolism in *Tetrahymena* or any other ciliate has been reported. To tackle this issue, a genome-wide survey of putative genes implicated in sphingolipid metabolism has been conducted in *Tetrahymena thermophila* with the aim of beginning to disclose the biosynthetic and phylogenetic pathways in this organism. Furthermore, we pinpointed a sphingolipid fatty acid 2-hydroxylase in the ciliate by somatic knockout and assessed its physiological role, which was found to significantly impact on the sexual stage of conjugation.

Results

Identification of genes involved in sphingolipid metabolism in *T. thermophila*.

To analyze the genetic diversity of the *T. thermophila* sphingolipid metabolic pathway (SMP) we decided to gather information using genomic data retrieved from the Tetrahymena Genomic Database (TGD) and the OMA orthology database. Based on the very few reports on sphingolipid species found in *T. thermophila,* we inferred the SMP including sphingolipid moieties reported in other species of *Tetrahymena*, particularly *T. pyriformis* and *T. mimbres*. Using the KEGG sphingolipid metabolic pathway map as a template we were able to identify 38 putative genes, corresponding to 11 enzymatic steps involved in sphingolipid biosynthesis and degradation in *T. thermophila* (Fig. 1 and Table S1). The evolutionary conservation of the enzymes shows a broad different degree ranging the highest values between 32,48 % - 54,90 % of similarity and 16,11 % - 41,25 % of identity, depending on the enzyme type and the evolutionary distance between different organisms (Fig. S1 and Table S2).

The genes identified in the *T. thermophila* genome belong to a *de novo* biosynthetic pathway of ceramides, sphingosine, and sphingosine-1-phosphate. In the case of the first biosynthetic enzyme, the serine palmitoyltransferase (SPT), two orthologous genes of the *H. sapiens* SPTC2 were retrieved from OMA. Interestingly, phylogenetic studies indicate that both genes, as well as other ciliates genes, could be acquired from related prokaryotic SPT (Fig. S1). These sequences are grouped into a branch which includes the characterized SPT from the *Sphingomonas_paucimobilis* (Ikushiro et al. 2001) and distant from others eukaryotic characterized SPTs from animals, plants, and fungi. Prokaryotic HGT events have been previously reported in the apicomplexan SPTs (Mina *et al*., 2017), therefore, the SPTs could have been acquired before ciliates and apicomplexans diverged from the alveolate common ancestor. Another possibility is that one of the *T. thermophila* sequence (Q22BA8) codifies for another enzyme since the most similar reviewed proteins (phylogenetic tree) codifies for an 8-amino-7-oxononanoate synthase involved in biotin biosynthesis. No clear orthologous of the second biosynthetic enzyme 3-Ketodihydrosphingosine reductase (KDHR) was found neither in KEGG nor OMA. Nevertheless, nine putative genes member of the short-chain dehydrogenase/reductase (SDR) superfamily which groups to the KDHR among other enzyme families, were retrieved using reviewed enzymes from animals, fungi, plants, and slime molds in TGD. This could imply that probably the ciliate use a different SDR type to the canonical 3-

dehydrosphinganine reductase to perform the conversion of 3-dehydrosphinganine to dihydrosphinganine (sphinganine). The third biosynthetic step is carried out by the ceramide synthase, which catalyzes the formation of dihydroceramide from dihydrosphingosine and acyl-CoA substrates. Using reviewed proteins from the plant *Arabidopsis thaliana* (LAG1 and LAG2), four *T. thermophila* orthologous were retrieved from OMA. The last step involved in de novo ceramide biosynthesis is the dihydroceramide desaturase. This enzyme, belonging to the fatty acid desaturase superfamily, catalyzes the Δ4 desaturation of dihydroceramide to generate ceramide. Two orthologous genes were retrieved from OMA using the *H. sapiens* enzyme (DEGS1). The synthesis of sphingosine and sphingosine 1P from ceramide involves two enzymes: a ceramidase (acid, basic or neutral) and a sphingosine kinase. Six orthologous of the acid ceramidase type and two of the sphingolipid kinase were retrieved from the KEEG database.

Previous studies reported ceramides moieties with n-C16, iso-C17 and n-C19 long chain bases and 16:0, iso-17:0 and 2-OH16:0 fatty acids in *T. pyriformis* (Kaya et al. 1984a). In contrast, the presence of free sphingosine and S1P was not reported in the *Tetrahymena* genus, probably due to very low levels in the cell (30 times less of sphingosine and 3000 times less of S1P than ceramides) (Hannun & Obeid 2008). The identification of two paralogs of sphingosine kinase in the macronuclear genome *T. thermophila* is in agreement with previous reports on the characterization of two forms of this enzyme in *T. pyriformis,* albeit with different activities and responses to inhibitors (Wang, Banno & Nozawa 2002).

Other sphingosyl phosphatides identified in *T. pyriformis* are sphingomyelin and the ceramide-phosphono-analogs, ceramide N-methyl-2-aminoethylphosphonate (CMAEP) and ceramide 2-aminoethylphosphonate (CAEP) (Sugita et al. 1979). Unexpectedly, no orthologous genes involved in the head group transfer to ceramide, such as the sphingomyelin synthases (SMS1/2), ethanolamine-phosphorylceramide synthase (EPC) or inositol-phosphorylceramide synthase (IPCs) were identified in the ciliate using as query ceramide synthases from organisms of diverse evolutionary origin. This could imply the presence of another enzyme involved in the phosphonate transfer to ceramide, not yet identified. With regard to the biosynthesis of ceramide 1 phosphate, we could not find any reports in the literature on the presence of this sphingolipid nor an orthologous gene of a ceramide kinase, ruling out the production of this bioactive sphingolipid in the cell.

The elucidation of the biosynthetic pathway of glycosphingolipids in *T. thermophila* is still a challenge. No putative orthologous genes of the glucosylceramide synthase, implicated in the first step of glycosylceramide biosynthesis in animals, fungi and plants (UGCG, HSX11, and At2g19880 respectively) were identified in the *T. thermophila* genome. Neither could we find orthologous genes of the glucosyltransferases nor the galactosylceramide synthase identified in animals. Genes involved in the metabolism of glycosylinositol phosphorylceramides, present in fungi, protozoans, and plants, were not identified in the ciliate as well. The only glycosphingolipid reported in the *Tetrahymena* genus is a glycosylphosphatidylinositol-anchored protein from *T. mimbres* which contains a ceramide with the long-chain base 3-O-methylsphinganine bound to palmitic and stearic fatty acids (Hung, Ko & Thompson 1995). The pathway to biosynthesize GPIs containing inositol-phosphorylceramide, the lipid remodeling pathway, involves the substitution of the diacylglycerol by a ceramide by three enzymes: a phospholipase A2, an O-acyltransferase and a ceramide transferase. The only genes retrieved in our search were three paralogs to the O-acyltransferase GUP1p from *Saccharomyces cerevisiae* (Bosson 2006).

The search of putative genes codifying for enzymes involved in the catabolism as well as the recycling and interconversion of sphingolipids such as sphingomyelinases, acid ceramidases, β-glucosylceramidases, and S1P phosphatases, retrieved only five paralogs of the human acid sphingomyelinases (*SMPD1*) and six paralogs of the human acid ceramidase (*ASAH1*), as commented above. In the case of the sphingosine-1-phosphate lyase, the final enzyme in the sphingolipid catabolic pathway and the only known exit from the sphingolipid metabolism, we could retrieve three paralogous sequences in the *T. thermophila* genome.

Finally we also searched orthologous genes of enzymes that introduce variations to the long-chain base structure such as the C4-sphingolipid hydroxylase and the delta-4-sphingolipid desaturase, the delta8-sphingolipid desaturase (these present in fungi and plants), and the C9-sphingolipid methyltransferase (only present in fungi), as well as enzymes that introduce variations to the N,-linked fatty acids such as the delta-3-sphingolipid desaturase (present only in some fungi) and the sphingolipid fatty acid 2-hydroxylase (present in most eukaryotes). The retrieved search showed only two paralogous genes for the delta-4-sphingolipid desaturase (commented above) and no other putative genes for other enzymes that modify the long-chain base. Regarding the enzymes that modify

the N-linked fatty acids, four paralogs of the delta-3-sphingolipid desaturase and two of the fatty acid 2-hydroxylase were identified in the *T. thermophila* genome. In the case of the latter, the identification and characterization of the enzyme activity have shown a specific requirement of the ceramide moiety as substrate, not replaceable by free fatty acids (Kaya, Ramesha & Thompson 1984b).

Identification and localization of FA2H from *T. thermophila.*

In order to reveal the gene network involved in sphingolipid metabolism in *T. thermophila*, we started by identifying the fatty acid 2-hydroxylase enzyme. FA2H is a membrane-bound oxidase that introduce the hydroxyl group to the α-carbon of the FA moiety of the ceramide substrate by coupling the electron-transport proteins NADH–cytochrome b_5 reductase and cytochrome b_5. The enzyme belongs to the FAH superfamily, which is characterized by the presence of conserved histidine motifs (Shanklin, Whittle & Fox 1994) and the presence of 4 membrane-spanning helices, as revealed by the crystal structure (Zhu et al. 2015).

Two putative sequences (UniProt ID: Q23PP8 and Q232H4) in the *T. thermophila* genome (Fig. 2A) were identified by Blast and orthology analyses using as query reviewed (Swiss-Prot) FA2H proteins from plants, fungi and metazoan. Both sequences contains a fatty acid hydroxylase domain (PF04116), as all members of this group of enzymes, and a cytochrome-b_5 domain (PF00173), which is present in many but not all FAHs of different organisms (Fig. 2B).

The search of other ciliate sequences using 8 ciliate genomes from different genera, retrieved putative sequences from *Euplotes octocarinatus*, *Oxytricha trifallax*, *Stylonychia lemnae* (classes Spirotrichea) and from *Stentor coeruleus* (class Heterotrichea), while other sequences from oligohymenophorean ciliates were not retrieved (Fig. 2C).

Phylogenetic analysis of FA2H proteins grouped both sequences into a well-defined monophyletic branch along with other putative FA2H proteins from spirotrichean and heterotrichean ciliates and closer to fungus than to archaeplastid and animal sequences (Fig. 2D).

Sequence analysis of the two putative proteins revealed that the Q232H4 sequence lacks the first two of the four characteristic histidine motifs of this enzyme family (HXXXXH, HXXHH, HXXXH, and HXXHH). These histidine motifs contain histidine residues that coordinate two iron atoms at the active center of the enzyme and are essential for catalysis. Single mutations of each of the four

histidines, which are absent in the Q232H4 sequence, have shown to alter the FA2H activity in *S. cerevisiae*. Furthermore, the conserved Asp-323, which is a critical determinant involved in the hydroxylase activity reaction through the stabilization of the ceramide headgroup, is replaced by glutamic acid (Fig. S2) (Zhu et al. 2015). These conserved histidine residues are critical not only in FA2H but in all enzymes belonging to the fatty acid desaturase and fatty acid hydroxylases superfamilies (Shanklin, Whittle & Fox 1994). Therefore, considering these critical modifications, we discarded the Q232H4 sequence for the analysis as a putative fatty acid 2 hydroxylase.

To evaluate whether Q23PP8 is a fatty acid 2-hydroxylase, a KO mutant strain (KOFA2H) was generated by eliminating the coding gene through a somatic knockout procedure (Fig. 3A-C). Growth and morphology during vegetative growth were indistinguishable between the WT and KO, suggesting that the gene (*FA2H)* is nonessential. The fatty acid composition of sphingolipids from both strains grown at 30°C is shown in Table S3. The relative amount of main fatty acid species in WT strain followed the order 2-OH 17:0 > 2-OH 18:0> 16:0 > 18:0 > 17:0. In contrast, the main fatty acids from the KOFA2H strain grown in the same medium and at the same growth temperature were 17:0, 18:0 > 16:0, and no fatty acids with a hydroxyl group at the α-carbon position were detected (Fig. 3D). The lack of residual activity in this mutant corroborates that the discarded Q232H4 sequence is not a bona fide fatty acid 2 hydroxylase.

We also measured the composition of lipids in membranes from WT and KOFA2H strain. Although no significant changes were observed in the composition of neutral lipid, the KOFA2H strain showed a different profile in the polar lipid fraction (Fig. 3E). In order to gain further insight into some lipid identification of the KOFA2H strain, we enriched different polar lipid fractions and analyzed by high resolution mass spectrometry the lipid moieties only present in the mutant strain (see Material and Methods). By this strategy, we could identify in the chloroform/methanol (70/30) fraction a precursor ion with a m/z of 645.5030 that is not present in the WT strain. The search in Lipid Maps and METLIN databases retrieved a potential compound with a formula of C36H73N2O5P which might correspond to an N-palmitoyl-D-sphingosyl-1-(2-aminoethyl)phosphonate. The main fragments (mz/mz) of 520.5026 and 502.4905 (I 100 % and 63 % respectively) generated correspond to N-palmitoylsphingosine (Fig. 3F) and are in agreement with the ceramide 2-aminoethylphosphonate identified.

In order to localize the enzyme in *T. thermophila* cells, the fatty acid 2 hydroxylase was C-terminally tagged with EGFP. The tagged gene was targeted to its endogenous locus (Fig. 3E) to avoid potential misexpression artifacts in the ciliate. As a result of the high autofluorescence of the background, remarkable even in wild type cells, the selectivity of the signal was increased using anti-GFP antibody and indirect immunofluorescence microscopy. The microscopic analysis of FA2H-EGFP revealed a strong signal around the nucleus, in a pattern that is consistent with a perinuclear localization, along with a more diffuse one in the cytoplasm (Fig. 4).

Biological role of FA2H from *T. thermophila.*

To get information on the potential biological role of the FA2H, the gene expression profile during the three major stages of the organism's life cycle (growth, starvation, and conjugation) was retrieved from the TGD and examined.

Considering that previous research works reported a significant increase in the transcription of the gene during the first hours of the conjugation stage, the behavior of WT and KO strains was analyzed (Fig. S3) during the entire conjugation sexual process. *T. thermophila* cells can have one of seven different mating types, and each mating type can only conjugate with a cell of another mating type, but not with the same (Elliott & Hayes 1953) (Fig. 5A). For the purpose of the analysis, we generated two fatty acid 2-hydroxylase KO mutant strains of different mating types, B2086.2, and CU427.4, as well as a non-related C5 sterol desaturase KO mutant strain (KOC5DES) in the CU428.2 WT strain, to be used as control. As shown in Fig. 5B, the cell pairing (early event in conjugation) decreased by around 88 % when a WT strain was induced to conjugate with any of the knockouts KOFA2H strains. In the case of conjugation between two KOFA2H strains of different mating types, no cell pairing events were observed at all. In contrast, conjugation between two WT strains or WT plus KOC5DES strain showed similar rates in pairing (64 % and 67 % respectively). We also analyzed the ratio of cells that completed the conjugation process (using phenotypic assays) concerning those that begin the cell pairing event (efficiency). It was shown that, in the case of the conjugation between a WT and a KOFA2H, only a very low percentage of conjugants were able to complete all the stages (1 %), whereas between the two KOFA2H strains of different mating type, no conjugants were recovered. Moreover, the efficiency between two WT strains or between one WT strain and one KOC5DES strain was similar (94 % and 92 % respectively). Additionally we measure the number of conjugating

cells and the progression of the meiotic stages every hour in WT x KOFA2H and compare to WT x WT cross. As shown in Figure 5C, along the first hours of conjugation (shown from 2 h to 5 h) it was observed a delay in the meiotic prophase stage I (significant at 2 h), stage IV (significant at 4 h) and stage 5 (at 5 h) in WT x KOFA2H, possibly as a consequence of the delay in the cells pairing.

Discussion

Over the past several years, studies on sphingolipids and their function as bioactive lipids have intensified in different model organisms, revealing a vast complexity of the lipidome and its many functions (Hannun & Obeid 2017). The genes implicated in the sphingolipid metabolism pathway have been identified in diverse eukaryotic lineages such as the Opisthokont clade including vertebrates, invertebrates and many fungi (Acharya & Acharya 2005; Merrill 2011; Montefusco et al. 2014); in the Archaeplastida clade including lower vascular plants and several species of higher plants (Michaelson et al. 2016); in the Kinetoplastida clade including *Trypanosoma* and *Leishmania* (Smith & Bütikofer 2010; Zhang & Beverley 2010) and in the apicomplexan clade including parasites *Plasmodium*, *Toxoplasma* and *Cryptosporidium* (Pratt et al. 2013; Ramakrishnan et al. 2013). The study of the sphingolipid metabolism pathway in *T. thermophila* performed in this work indicates that both conserved and innovative mechanisms are used in ciliates compared with those in other eukaryotic lineages. For example, the genes involved in the head group transfer to ceramides, such as the SMS1/2, EPC or IPCs which at least one of them is present in all the eukaryotic organism studied up to date (Tafesse, Ternes & Holthuis 2006) were not identified in the ciliate, suggesting an innovative mechanism for the phosphonate-group transfer to ceramides. Genes involved in glycosphingolipid biosynthesis (excluding the lipid remodeling pathway) were not retrieved in our search. Although this can suggest a different and innovative mechanism in ciliates, the fact that only a few genes have been presently identified in most other organisms, i.e., kinetoplastids and apicomplexans, indicate that further studies are required to support this hypothesis. Moreover, the presence in the ciliate of orthologs of many conserved enzymes in other organisms such as the serine palmitoyltransferase, 3-ketodihydrosphingosine reductase, dihydroceramide synthase, dihydroceramide C-4desaturase, sphingosine kinase, and sphingosine-1-phosphate lyase reinforces the concept of conservation of the general sphingolipid metabolism pathway in the eukaryotic kingdom.

The genome analysis also allowed the identification of two putative enzymes introducing variations to the basic sphingolipid structure, such as the fatty acid 2-hydroxylase. Using a KO strategy, we demonstrated that the TTHERM_00463850 gene codes for a fatty acid-2 hydroxylase in the ciliate *T. thermophila*. Lipid analysis of *Tetrahymena* membranes from KOFA2H mutant strain, identified at least one sphingolipid moiety, N-palmitoyl-D-sphingosyl-1-(2-aminoethyl) phosphonate, not present

in the WT strain. Although different ceramide 2-aminoethylphosphonate have been previously identified, to the best of our knowledge, this is the first identification of a ceramide 2-aminoethylphosphonate with both the detailed composition of the N-acyl fatty acid as well as the sphingoid long chain base within the same molecule.

The fatty acid-2 hydroxylase is the third enzyme belonging to the FAH superfamily that was identified and characterized in the ciliate, together with a C5 sterol desaturase and a C24 sterol deethylase (Nusblat et al. 2009; Tomazic et al. 2011); and, so far, the first one involved in sphingolipid metabolism. The FAH enzymes are integral membrane proteins that are located in the endoplasmic reticulum, which is in accordance with the ER localization of the FA2H-EGFP fusion in the ciliate and is consistent with the localization of other FAHs studied in *T. thermophila* (Poklepovich et al. 2012) as well other FA2H from yeast (Natter et al. 2005), mammalian (Eckhardt et al. 2005) and plants (Nagano et al. 2012).

Phylogenetic analysis grouped the *T. thermophila* FA2H in a monophyletic cluster with other putative FA2H proteins from spirotrichean and heterotrichean ciliates. It is worth noting that no orthologous genes have been retrieved from the genome of other oligohymenophorean ciliates such as *Ichthyophthirius multifiliis*, *Pseudocohnilembus persalinus,* and *Paramecium tetraurelia*. In the case of the *I.multifiliis* and *P. persalinus,* the absence of the gene could be a result of their parasitic lifestyle. Actually, these ciliates have lost many genes during their extensive genome reduction (Xiong et al. 2015). However, the absence of the gene in the free living *P. tetraurelia* is not clear, much less, when 2-hydroxy fatty acids sphingolipids have been reported in lipid extracts of the ciliate (Kaneshiro 1987). Notably, unlike *Tetrahymena*, which has hydroxy fatty acids in its ethanolamine sphingophosphonolipids, in *P. tetraurelia* hydroxyl fatty acids were found only associated with glycosphingolipids, characterized by a fatty acid composition with high concentrations of C20 to C24 neutral and a long chain of hydroxy fatty acids, suggesting that the free ceramides are not direct precursors of the glycosylated ceramides and that another synthesis pathway may be involved (Kaneshiro et al. 1997).

2-hydroxy ceramides, synthesized by the FA2OH, have been shown to stabilize the myelin of the mammalian nervous system. It was observed that mutations in this gene are associated with various types of neurodegeneration, including brain iron accumulation, leukodystrophies, and hereditary

spastic paraplegias. 2-hydroxy ceramides also regulate the differentiation of various cell types (epidermal keratinocytes, schwan-noma cells, adipocytes, megakaryocytic cells) and have been showed to mediate proapoptotic signaling distinct from non-hydroxy ceramide, as well as the involvement in the regulation of gastric tumor growth (Hama 2010; Yao et al. 2019). The enzyme has been also studied in the yeast *Saccharomyces cerevisiae* where it was shown to be non- essential for growth (Haak et al. 1997), in the plant Arabidopsis thaliana where it was shown to be involved in the resistance to oxidative stress (Nagano et al. 2012) and in the invertebrate *Caenorhabditis elegans* in which it was shown to affect intestinal homeostasis, by interfering with heptadecenoic acid production (Li et al. 2018). Alternatively, our experiments demonstrated that growth and morphological parameters were not significantly different between KO and WT strains, suggesting that the gene is nonessential during *T. thermophila* vegetative growth. However, our results revealed a severe impairment in the sexual stage of conjugation. This process requires starved pairs of sexually mature cells with different mating types to begin to establish contact with each other (cell-cell contact, referred to as co stimulation) to form a stable pair. The cells then undergo a variety of highly conserved processes including meiosis, mitosis, nuclear exchange and karyogamy, and two postzygotic divisions of the fertilization nucleus that last 12 hours (Cole & Sugai 2012). The fail to form cell pairs when two KOFA2H strains of different mating type were induced to conjugate, suggests a deficiency during the early process of co-stimulation. This process involves a tip transformation of the cells, with a reorganization of surface glycoproteins (0–30 min after mixing different mating type starved cells), and a progress in pairing from loose to tight that finally establishes a mating junction (30-120 min) (Cole 2016). As previous studies have demonstrated, lipid domains are formed and re-arranged during this process in response to this structural requirement, including cylindrical lipid species replacement at the exchange junction with nonlamellar species supporting a tight curvature during conjugation (Ostrowski et al. 2004; Kurczy et al. 2010). Hypothetically, the lack of 2-hydroxy sphingolipids in the KOFA2H strains could be affecting the correct reorganization of surface glycoproteins associated with lipid rafts, usually enriched in sphingolipids, or it could affect the stabilization of lipid domains as was reported in the myelin of vertebrates (Simons & Ikonen 1997). In our experiments, a low percentage of WT x KOFA2H cells were able to form cell pairs, indicating that some reorganization of surface glycoproteins could take

place. Nevertheless, the number of cells that completed the conjugation process in this cross was negligible, suggesting that, although a low number of pairs make new micronuclei, they may abort during macronuclear development, a stage that resembles round one of genomic exclusion,(Martindale, Allis & Bruns 1982). Another possibility that could explain the differences in numbers between pair formation and conjugation completion could be the use of different counting methodologies, especially when starting with a small number of pairing cells, as in the case of WT x KOFA2H pairs (see Materials and methods).

The impairment in finishing the conjugation process in the ciliate resembles the mating-specific yeast cell cycle arrest caused by the deficiency in ceramide moieties with very long chain fatty acids (Villasmil et al. 2017).

To sum up, the current results highlight the significance of the FA2H in the cell pairing process, a step that is essential in the conjugation stage of *T. thermophila*, as well as the suitability for using this ciliate as a valuable research model to investigate sphingolipid metabolic pathways and their evolutionary conservation and divergence in the eukaryotic lineage.

Experimental Procedures

Strains, plasmids, and *T. thermophila* growth conditions

T. thermophila strains CU428.2 *mpr1-1/mpr1-1* (*MPR1*; mp-s, VII), B2086.2 (II) and CU427 *chx1-1/chx1-1* (CHX1; cy-s, VI), designated as "wild type", and plasmids pBS-MnB-3 and pmEGFP were retrieved from the Tetrahymena Stock Center (Cornell University, NY, USA). *T. thermophila* cultures were grown in PPYE medium (1% Proteose peptone (Britania, Argentina), 0.1% Yeast extract (Britania), 0.5% Glucose (Britania) and 0.003% Iron (III) citrate (Sigma-Aldrich, USA)) at 30°C and 80 rpm in 250 mL Erlenmeyer flasks. Paromomycin (Sigma-Aldrich) was added from a 200 mg/ml PPYE stock solution when indicated, along with 1 μg/ml of $CdCl_2$, prepared as a 1 mg/ml stock solution in water. A 1:10 dilution of a 24-h culture was inoculated daily into the cultures. Cells were counted in a Neubauer chamber (Cassidy-Hanley 2012). To perform the mating assays, cells of different mating types were starved for 16-20 h and 5 ml of each culture were mixed in a 100 x 15 mm petri dish to yield a 1:1 ratio at a cell concentration of $1x10^5$ to $2x10^5$ cells/ml. Cell pairing was recorded at 4hs after mixing. At 30 hs after mixing (time necessary to complete the conjugation process and allow the accumulation of ribosomes with the resistant cycloheximide version) the cells were distributed into 96-well plates (100 ul/well) varying the number of cells/well in ten fold dilution. Later, 2x PPYE media was added to the half of the plates (100 ul/well), while 2x PPYE media with 25 μg/ml of cycloheximide was added to the rest (100 ul/well). The rate of cells that completed conjugation process was estimated as the ratio between the number of wells grown in media with and without cycloheximide (%) (Hamilton and Orias, 2000; Chalker, 2012).

Standard DNA and RNA manipulation procedures for PCR and RT-PCR

Genomic DNA from *T. thermophila* CU428 (WT strain) was prepared as previously described (Cassidy-Hanley et al. 1997). Isolation of plasmid DNA from *E. coli* was performed using a QIAprep Spin Miniprep DNA purification system kit (Qiagen, Germany). Total RNA was prepared from *T. thermophila* cultures using TRIzol reagent (Invitrogen, CA, USA). Nucleic acid fragments were amplified by PCR using FIREPol DNA polymerase (Solis Biodyn, Estonia) or KAPA HiFi DNA Polymerase (Kapa Biosystems, USA) when high fidelity amplifications were needed. Moloney

murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA) was used to conduct reverse transcription (RT) reactions following the manufacturer's instructions.

Construction of FA2H and C5 Sterol desaturase KO strains, and FA2H-EGFP fusion in *T. Tetrahymena*

For FA2H gene disruption in *T. thermophila*, the transformation sequence FA2H-neo3 was constructed. The 753 bp upstream (Up) and 801 bp downstream (Dw) flanking regions of the putative sequence TTHERM_00463850 (Q23PP8) were synthesized separately (Genscript, USA). Both sequences were cloned into plasmid pBS-MnB-3, which confers paromomycin resistance and transformed into competent *E. coli* DH5α cells (Shang *et al.* 2002). Then, FA2H-neo3 construction was released by *KpnI* and *SacI* double restriction enzyme digestion and then concentrated by ethanol precipitation. The transformation sequence FA2H-EGFP was constructed for localization of FA2H in a *T. thermophila* cell. A 912-base pair (bp) C-terminal fragment of the FA2H gene (Up), without the stop codon, was amplified by PCR using specific primers adding *Bam*HI and *Sac*I restriction sites at the ends of each fragment. Cloning of the sequence into pmEGFP (confers paromomycin resistance) was performed (Kataoka *et al*. 2010). Next, the amplification of downstream flanking region of 917 bp (Dw) was also performed with the addition of *Xho*I and *HindIII* restriction sites at the ends of each fragment and cloned into pmEGFP+Up. For C5 sterol desaturase gene disruption, the flanking regions of the TTHERM_01194720 sequence were cloned into plasmid pBS-MnB-3 as detailed in Nusblat et al. 2009. The FA2H-EGFP construct was then released with *SacI* restriction enzyme from the vector. For *T. thermophila* transformation, we grew B2086 strain cells in 30 ml of PPYE medium at 30°C to reach a density between 2,5-3x10^5 cells/ml. Cultures were starved overnight in 10 mM Tris HCl buffer pH 7.5 and transformed with 10-15 μg of linearized FA2H-neo3 or FA2H-EGFP constructs by a biolistic-gun protocol (Chalker 2012). Bombardment was performed with a Dupont biolistic PDS-1000/He particle delivery system (Bio-Rad). Transformants were allowed to recover in 50 ml PPYE for 4 h. After this period, the cell suspension was diluted with 25 ml pre-warmed PPYE, to which paromomycin, cadmium chloride and an antibiotics/antifungal cocktail (streptomycin 250 μg/ml, penicillin G 250 μg/ml and amphotericin B 1 μg/ml) were added, at a final concentration of 0.12 mg/ml paromomycin and 1 μg/mL $CdCl_2$, and the entire culture was distributed in 96-well culture plates for incubation. The selection procedure was conducted by repeatedly transferring cells into

cultures with 1 μg/mL $CdCl_2$ and increasing paromomycin concentration until no further growth was achieved (70 mg/ml, 55 mg/ml and 45 mg/ml for the C5 sterol desaturase KO, KOFA2H and FA2H-EGFP fusion respectively).

Lipid extraction and identification by gas chromatography and mass spectrometry

We collected *T. thermophila* cells from cultures grown in Erlenmeyer flasks at 30°C (2.0-4.0 $x10^5$ cells/ml) by centrifugation at 1500 *g* for 5 min, and the extraction of total lipids was performed by the Bligh and Dyer method (Bligh & Dyer 1959). For analysis of the N-linked fatty acids, the organic phase was evaporated under N_2 stream and saponified with 2 ml of NaOH 1,5N in methanol 2 h at RT. Samples were extracted with 2 volumes of $CHCl_3$ and 1 volume of H_2O. The organic phase was recovered and evaporated under N_2 stream and resuspension of the residue was performed in 1 ml of $CHCl_3$. Each sample was seeded in a silica cartridge (Sep-Pak Vac 3cc) pre-conditioned with $CHCl_3$, washed with 10 ml of $CHCl_3$: AcH (9:1) and then eluted with 10 ml of MeOH to yield the sphingolipid fraction. Following evaporation under N_2 stream, the samples were resuspended in 10 ml of MeOH: HCl (5:1) and left overnight at 80°C. The methanolic phase was washed twice with 4 ml of hexane. The analysis of the composition of trimethyl-silyl-fatty acid methyl ester (TMS-FAME) derivatives was conducted in a Hewlett Packard HP 6890 gas chromatograph equipped with a Zebron ZB-5 column (30 m long, 0.53 mm inner diameter; Phenomenex, California, USA). The rise in column temperature was programmed at 10°C/min from 110 to 310°C and subsequently held for 10 min at 310°C. Mass spectrometry (MS) detection was performed with the use of an HP mass selective detector (Mass Spec Model 5975VL) operated at an ionization voltage of 70 eV with a scan range of 50–600 atomic mass units (amu). The identification of fatty acids was done by comparing them with NIST database standards (National Institute of Standards and Technologies, MD, USA). For analysis of intact lipid moieties by MS/MS, the Bligh and Dyer organic phase was evaporated under N_2 stream and separated by low pressure column chromatography in silica gel G 230-400 mesh, following the procedure detailed in (Hanahan 1997). Briefly, we performed the resuspension of the lipid extract in chloroform:methanol 6:1 and applied it on the column. After draining, the following step-wise elution was processed: acetone/methanol (90/10); chloroform/methanol (80/20); chloroform/methanol (70/30); chloroform/methanol (55/45) and methanol (100). Each fraction, as well as the Bligh and Dyer extract were analyzed by using silica gel G thin-layer chromatography (TLC) (Nozawa &

Thompson 1971). For polar lipid, the elution was carried out with chloroform: acetic acid: methanol: water (75: 25: 5: 2.2, v/v) and detected with Dittmer reagent. For neutral lipids the elution was carried out with hexane:diethylether:acetic acid (70:30:1, v/v) and detected with iodine vapors. Determination of the exact mass of lipid molecular species from eluted fractions was performed by using a Bruker micrOTOF-Q II mass spectrometer equipped with an ESI source set to positive ionization mode. MS data were acquired within the mass range of m/z 300–1,100. The ESI parameters used were: capillary 4.5 kV; end plate offset, -500 V; dry heater, 200°C; dry gas flow, 4.0 l/min; nebulizer 0.4 bar. For MS/MS, the collision energy used was 23.0 ev.

Microscopic Analysis

Tetrahymena cells expressing FA2H-EGFP were fixed with 2% paraformaldehyde in 50 mM HEPES pH 7.0 for 10 min at room temperature and permeabilized with ice-cold 0.1% Triton X-100 in 50 mM HEPES pH 7.0 for 10 min. After washing the fixed cells three times with ice-cold HEPES, they were treated with a blocking solution (1% bovine serum albumin in TBS buffer) at room temperature for 1 h and incubated in blocking solution containing anti-EGFP primary antibody (A-11122; Invitrogen) for 30 min at a 1:400 dilution. Cells were washed three times with TBS buffer containing 0.1% BSA for 5 min each and subsequently incubated at room temperature with goat anti-mouse-Alexa Fluor 594 secondary antibody (A-11020; Invitrogen) for 1 h at a 1:200 dilution in blocking solution. After one wash in TBS buffer containing 0.1% BSA, the cells were incubated with 1 mM DAPI (4′,6-diamidino-2-phenylindole, diluted in EtOH) at room temperature for 15 min. Finally, cells were washed twice with 50 mM HEPES pH 7.0 and mounted with anti-bleaching solution (0.2% n-propyl gallate, 90% glycerol in PBS). Digital images were collected using a Carl Zeiss Axio imager M2 fluorescence microscope. Images were analyzed with Image J 1.50i (Wayne Rasband, National Institute of Health, USA).

Bioinformatics and phylogenetic analyses

A dataset comprising the fatty acid 2-hydroxylases was generated by extensive sequence homology searches using as query sequences from representative eukaryotic FA2H proteins. Sequences were retrieved from the National Center of Biotechnology Information (NCBI), UniProtKB (EMBL-EBI) and from the genomes of the ciliates *E. octocarinatus* (http://ciliates.ihb.ac.cn/database/species/eo), *P.*

persalinus (http://ciliates.ihb.ac.cn/database/species/pp), *P. tetraurelia* (http://paramecium.cgm.cnrs-gif.fr/) and *I. multifiliis*, *S. coeruleus*, *O. trifallax*, *S. lemnae* and *T. thermophila* (http://ciliates.org). OrthoMCL database v5 and OMA browser were also used to retrieve the orthologous sequences from FA2H proteins. Redundant sequences were excluded from the dataset sequences using BLASTp tool at NCBI homepage with the default setting parameters (Substitution matrix: BLOSUM62; word size: 6; gap opening cost: 11; gap extension cost: 1). Pairwise alignments of each individual sequence from the dataset against the entire dataset were retrieved. Those sequences showing 100 % identity with another sequence from the dataset were considered repeated sequences and thus, were excluded from the final dataset. CD-HIT with a sequence identity cut-off of 0.99 was also used to exclude redundant sequences. For conducting the phylogenetic analyses, PROMALS3D with default parameters was used to obtain dataset multiple alignments (MSA). Poorly aligned sequence regions were removed using trimAl (http://trimal.cgenomics.org/) with the gappyout-tool. This MSA was used to obtain the best-fit evolution model using the ProtTest software (Darriba et al 2011) and the phylogeny was constructed by the maximum-likelihood method using PhyML software (Guindon et al. 2010).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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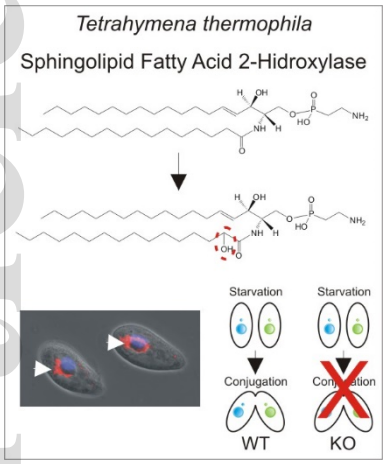
Author contributions

NGC: Data curation; Formal analysis; Writing - review & editing. CBN: Formal analysis; Conceptualization; Funding acquisition; Supervision; Writing - review & editing. ADN: Formal analysis; Conceptualization; Funding acquisition; Investigation; Supervision; Roles/Writing - original draft; Writing - review & editing.

Data Sharing

The data that support the findings of this study are available upon request.

Graphical abstract:



Abbreviated Summary

The putative sphingolipid metabolic pathway in *T. thermophila* was reconstructed.

Conserved and innovative mechanisms are used in the ciliate.

A fatty acid 2-hydroxylase was identified and characterized from *T. thermophila.*

ΔFA2H strains have shown impairments in the sexual stage of conjugation.

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Figures legends

Figure 1: Sphingolipid metabolism pathways of eukaryotic organisms, highlighting *Tetrahymena thermophila* pathways. Enzymes identified in the *T. thermophila* genome are shown in green and the sphingolipid moieties reported in the *Tetrahymena* genus are drawn. CAEP: ceramide 2-aminoethylphosphonate, S1P: sphingosine-1-phosphate, EPC: ethanolamine-phosphorylceramide, C1P: ceramide-1-phosphate, PPC : phosphonate-phosphorylceramide, IPC: inositol-phosphorylceramide.

Figure 2: A. Schematic representation of the phylogenetic tree, showing major eukaryotic supergroups and the position of phyla based on the phylogenetic analysis by He et. al. (He et al. 2014). Blue circles indicate the presence of fatty acid 2-hydroxylase previously identified and used as query for the search of other fatty acid 2-hydroxylases. The red circle shows the position of ciliates in the eukaryotic tree. **B.** Schematic representation of the secondary structure of a model of the *T. thermophila* fatty acid 2-hydroxylase placed within a model lipid bilayer (gray lines) is depicted. The protein structure was predicted using the I-TASSER server with default setting parameters (Yang et al. 2015). In dark grey the cytochrome-b_5 domain (at the upper part of the molecule) and the fatty acid hydroxylase domain (at the bottom part of the molecule) are shown in dark grey. **C.** Schematic diagram showing the orthologous sequences retrieved from ciliate genomes in green. **D.** Phylogenetic analysis of fatty acid 2-hydroxylase. For phylogenetic analyses, PROMALS3D with default parameters was used to obtain dataset multiple alignments (MSA). Poorly aligned sequences were removed using trimAl (http://trimal.cgenomics.org/) with the gappyout-tool. This MSA was used to construct the phylogenetic tree by the neighbor joining method with support of 1000 bootstraps using MEGA X software. Phylogenetic trees obtained with Minimum Evolution and Maximum Parsimony methods showed similar reconstruction. The S. cerevisiae C5-sterol desaturase (P32353) was used as outgroup. Black arrows indicate putative FA2H from *T. thermophila*.

Figure 3: A. Schematic representation of *FA2H* gene replacement in the wild-type (WT) locus, primers used to check the incorporation of neo 3 cassette in the *FA2H* locus are shown with arrows, grey for WT locus and black for knock-out (KO) locus. **B.** Genomic DNA of the *T. thermophila* WT and KOFA2H strains analyzed by PCR to check the gene replacement event. C. FA2H gene

transcription in WT and KOFA2H strains analyzed by RT-PCR. **D.** Fatty acid composition of sphingolipids from WT and KO strains expressed as a percentage of the total amount. Results are shown as the mean ± SD of three independent cultures. **E.** Polar lipids of WT and KO strains analyzed by TLC using chloroform: acetic acid: methanol: water (75: 25: 5: 2.2, v/v) as elution solvents. NL: neutral lipids, AEPL: ceramide 2-aminoethylphosphonate, PE:phosphatidylethanolamine, PC: phosphatidylcholine, SM: sphingomyelin. A red arrow indicates a polar lipid moiety present only in the KO strain. **F.** Mass spectra for the ceramide 2-aminoethylphosphonate identification. m/z obtained: precursor ion: 645.5330, fragments: 520.5026 and 502.4905. m/z of predicted ions: precursor ion: 645.5330 ([M+H]+), fragment m/z : 502.4940 ([M]+) and 520.5060 ([M+H]+).

Figure 4: Localization of EGFP tagged FA2H in *T. thermophila.* **A.** Schematic representation of gene replacement in the wild-type (WT) locus of the *FA2H* gene by targeting the FA2H-EGFP construct by homologous recombination, using a cassette in which neo 4 confers paromomycin resistance. **B.** Immunofluorescence localization of EGFP-tagged FA2H. WT and FA2H-EGFP strains were grown to Log phase and fixed for indirect immunofluorescence staining. The EGFP was localized by anti-GFP primary antibody followed by labeling with anti-mouse-Alexa Fluor 594 secondary antibody. The ciliate nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole, diluted in EtOH).

Figure 5: A. Stages of conjugation in *Tetrahymena.* **B.** Efficiency of the conjugation sexual process in WT and fatty acid 2-hydroxylase KO strains as well in the C5-sterol desaturase KO strain, used as control. The percentage of cell pairing and the percentage of them finishing the conjugation process are shown as the mean ± SD of three independent cultures. Statistical analysis was performed using one-way ANOVA. *** denotes statistical significances ($P \leq 0.001$, respectively) with respect to the value recorded in WT x WT conjugation assay. **C.** Temporal analysis of meiotic stages of conjugation between two WT strains; and one KOFA2H strain with one WT strain of a different mating type. Roman numerals indicates the six stages of meiotic prophase. A percentage of each meiotic stage from the total of cell pairings is shown in each hour. Between 42 and 84 cell pairings were examined in triplicate experiments. *** denote statistical significances ($P \leq 0.001$).

Supplementary information

Figure S1: Phylogenetic analysis of putative enzymes corresponding to enzymatic steps involved in the *T. thermophila* sphingolipid metabolism pathway. *T. thermophila* putative proteins are shown in blue, reviewed proteins (Swiss-Prot) in green, and proteins used as outgroups in red. To infer phylogenetic trees of each putative enzymes, different datasets comprising sequences retrieved by BLAST at UniProt were generated using as query the sequences listed in Table S1. Sequences with an E value below of 1e-10 were filtered. To reduce the number of sequences to approximately 80 to 120 in each dataset, we cluster proteins using CD-HIT at 40-80 % of sequence identity (Li and Godzik, 2006). Amino acid sequences were aligned using the MAFFT algorithm (Katoh and Standley, 2013) implemented in the online resource at CBRC, Japan (MSA Supporting Information). Phylogenetic relationships were determined using Maximum Likelihood using PhyML 3.0 optimized by SPR and NNI with a support of 100 bootstraps at the ATGC online resource. The evolutionary models for the MSA were selected using the Smart Model Selection method (SMS) (Lefort *et al*., 2017).

Figure S2: Sequence similarities of reviewed fatty acid 2-hydroxylases and putative sequences of *T. thermophila.* The four histidine clusters are indicated with a solid green line. The secondary structure of *S. cerevisiae* FA2H is shown at the top of the alignment (PDB 4ZR0). The conserved residues absent in the Q232H4 *T. thermophila* sequence, are indicated with green arrows for the histidine residues and in blue arrow for the aspartic acid 323 residue. Amino acid sequences were aligned using the MAFFT algorithm (Katoh and Standley, 2013) implemented in the online resource at CBRC, Japan. The figure was generated using the Espript program (Robert and Gouet, 2014).

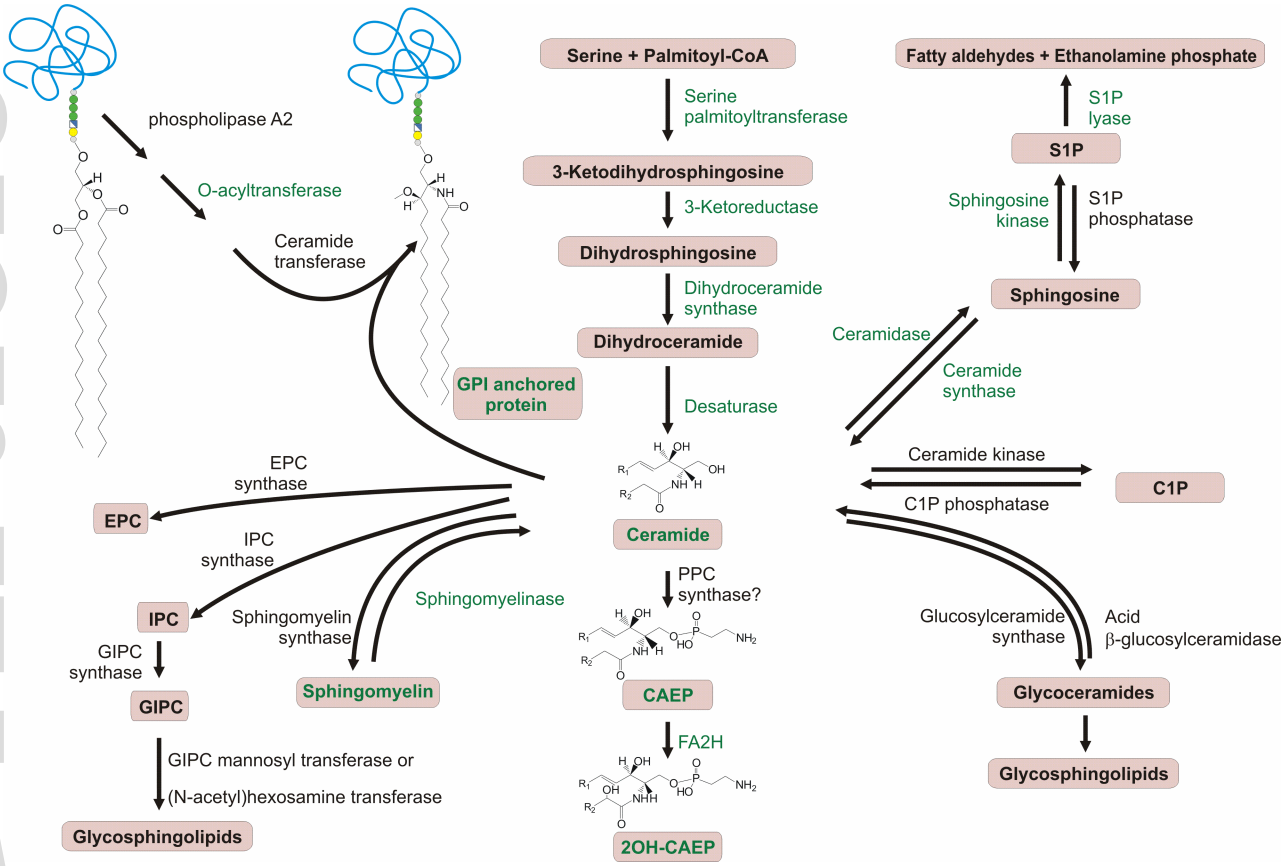
Figure S3: Gene expression profile of *FA2H*. Blue and red lines represent the expression values normalized by two different methods. For growing cells (L) l, m and h correspond respectively to 1x105 cells/ml, 3.5x105cells/ml and 1x106 cells/ml. For starvation (S) 2x105 cells/ml were collected at 0, 3, 6, 9, 12, 15 and 24. For conjugation (C) B2086 and CU428 cells were mixed, and samples were collected at 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 hours after mixing. Data were retrieved from the Tetrahymena Functional Genomics Database.

Table S1: Putative enzymes of *T. thermophila* sphingolipid metabolism pathway. The enzymes were search by sequence homology using query reviewed proteins (Swiss-Prot) of different organisms. To retrieve reliable sequences, the OMA (Orthologous MAtrix) database, the KEGG (Kyoto Encyclopedia of Genes and Genomes) sphingolipid metabolic pathway, and the Tetrahymena Genomic Database (TGD) were used. Putative sequences with an E value below of 1e-10 were filtered.

Table S2: Evolutionarily conservation of putative enzymes of *T. thermophila* sphingolipid metabolism pathway. The identity and similarity percentage of *T. thermophila* proteins with reviewed proteins (Swiss-Prot) from different organisms were calculated using the Ident and Sim program from the Sequence Manipulation Suite (Stothard 2000). The highest values are shown in green and blue (identity and similarity respectively).

Table S3: Fatty acid composition of sphingolipids from WT and KOFA2H strains grown at 30°C. Results are shown as the mean ± SD of three independent cultures. Statistical analysis was performed using one-way ANOVA. *, ** and *** denote statistical significances ($P< 0.05$, $P\leq 0.01$ and $P\leq 0.001$, respectively) with respect to the value recorded in WT cells.

MSA Supporting Information: Multiple sequence alignments in Fasta format used for phylogenetic reconstruction are included in a zip file.



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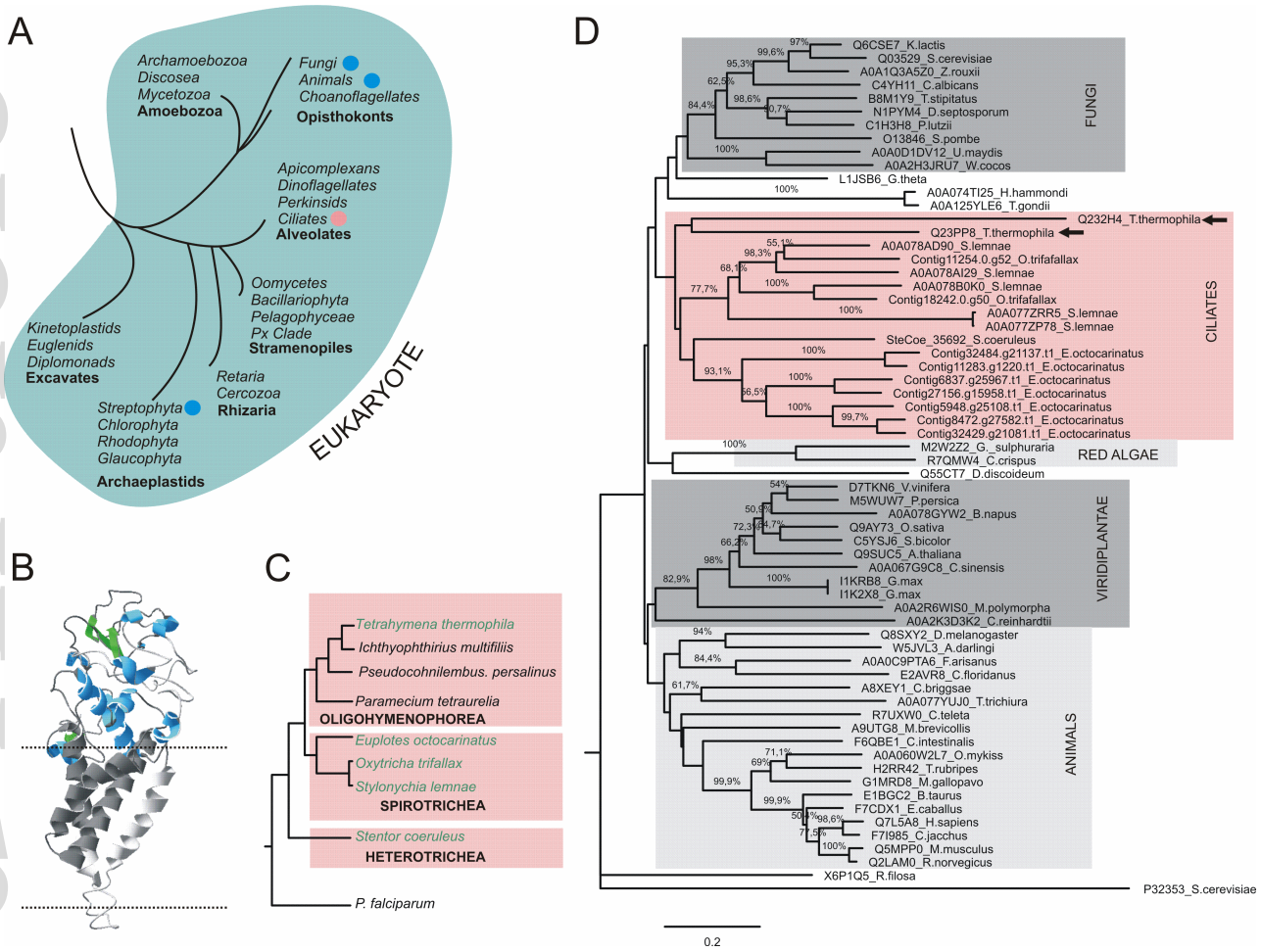

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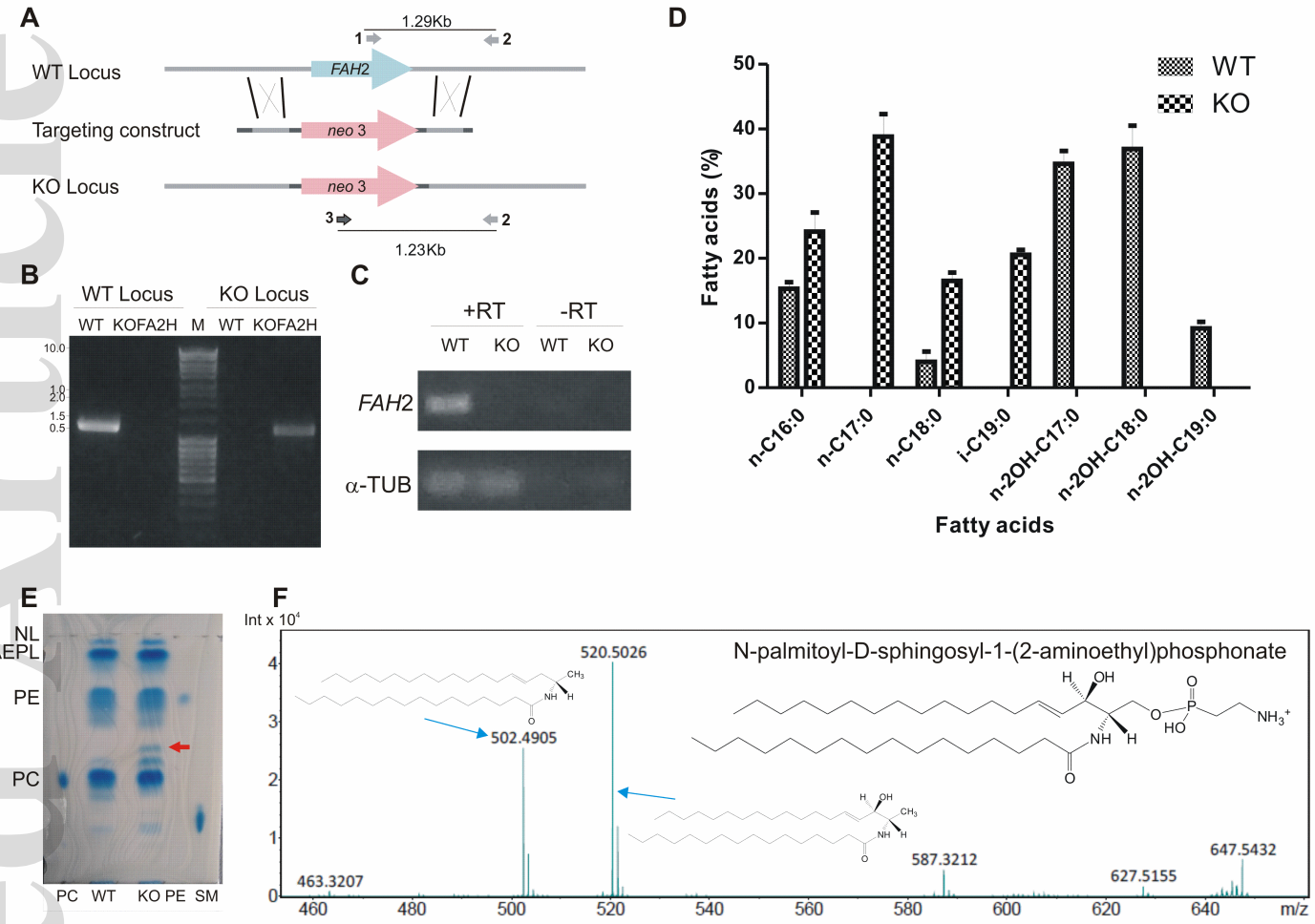

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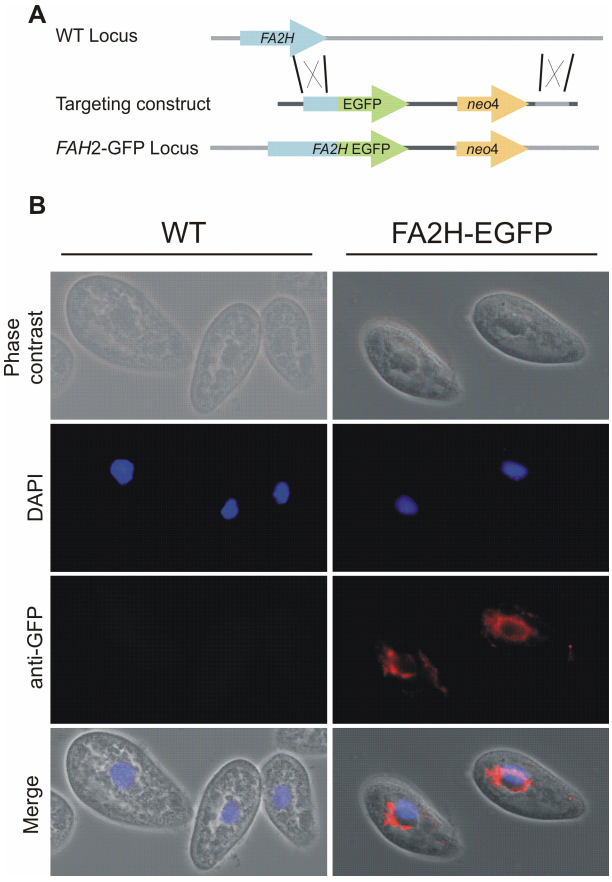

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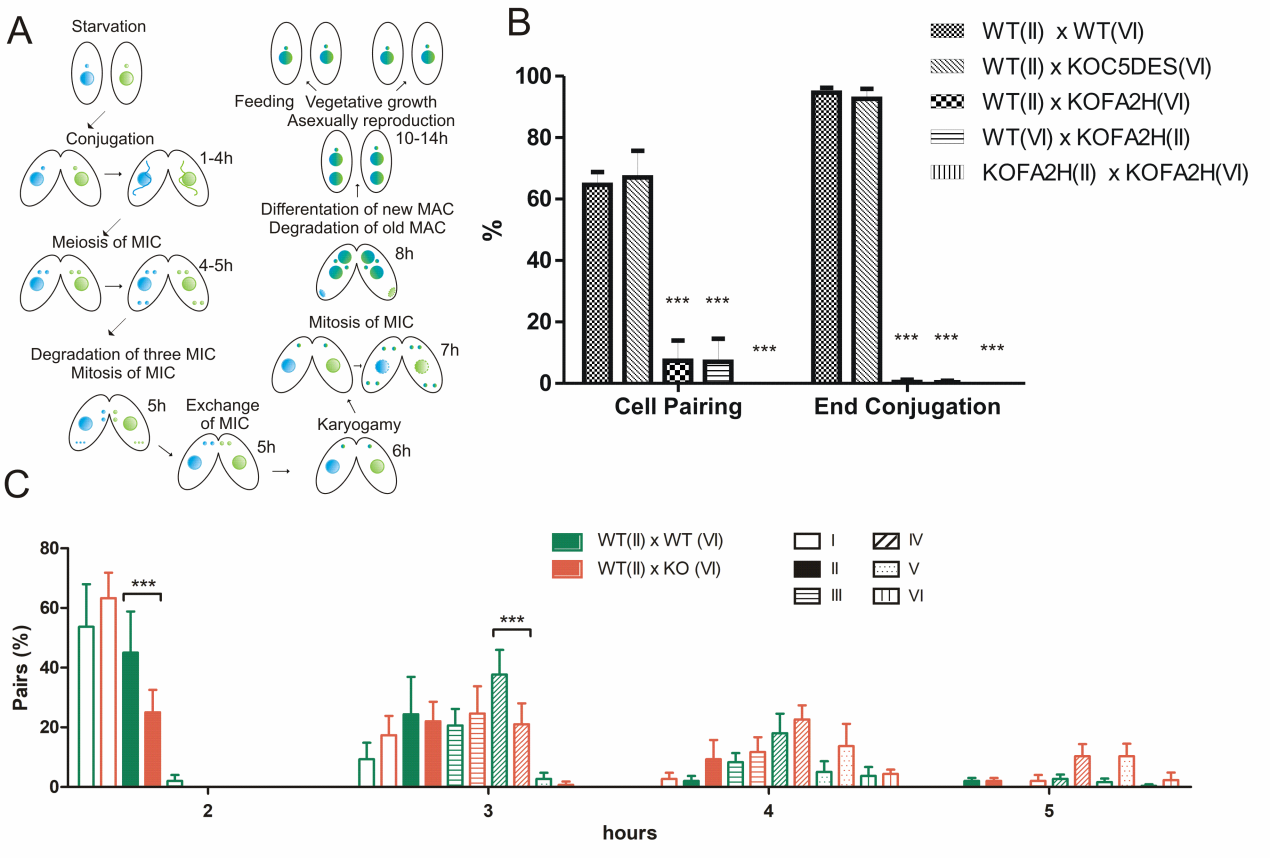

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