A fatty-acid synthesis mechanism specialized for parasitism

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Abstract | Most cells use either a type I or type II synthase to make fatty acids. *Trypanosoma brucei*, the sleeping sickness parasite, provides the first example of a third mechanism for this process. Trypanosomes use microsomal elongases to synthesize fatty acids *de novo*, whereas other cells use elongases to make long-chain fatty acids even longer. The modular nature of the pathway allows synthesis of different fatty-acid end products, which have important roles in trypanosome biology. Indeed, this newly discovered mechanism seems ideally suited for the parasitic lifestyle.

Glycosome
An organelle, related to peroxisomes, found in trypanosomatid protozoans that contain enzymes of the glycolytic and other metabolic pathways.

Trypanosomes and *Leishmania* spp. belong to the family trypanosomatidae and many members are parasites that cause human disease. *Trypanosoma brucei* is responsible for human sleeping sickness in sub-Saharan Africa, *Trypanosoma cruzi* causes Chagas' disease in Latin America, and *Leishmania* spp. cause various types of leishmaniasis in tropical and sub-tropical regions. Few drugs are available to combat these diseases and many that are available are toxic and susceptible to the development of resistance. The need for new drugs and drug targets dictates much of the research that is done on these organisms.

These flagellated protozoans branched early in evolution and therefore display significant deviations from standard eukaryotic paradigms. One such metabolic difference is that trypanosomatids confine glycolysis to a peroxisome-related organelle called the glycosome[1]. FIGURE 1 depicts this and other trypanosome organelles that are relevant to metabolism. Another remarkable feature, discovered only recently in *T. brucei*, is that these parasites synthesize most of their fatty acids (FAs) by an unprecedented mechanism[2]. This unusual mode of FA synthesis and how it contributes to the special biology of these parasites is the focus of this review. There are other recent reviews on *T. brucei* lipid metabolism[3,4].

The trypanosome life cycle

Trypanosomatid parasites adapt to multiple environments during their life cycle. Although *T. cruzi* and *Leishmania* spp. invade host cells, *T. brucei*, the species most frequently discussed in this review, multiplies extracellularly in the mammalian host blood. In advanced stages of human sleeping sickness, trypanosomes traverse the blood–brain barrier and invade the cerebrospinal fluid[5]. To transfer the infection to a new host, the long, slender bloodstream form (BSF) of the parasite differentiates into a short, stumpy form that is then ingested by the tsetse vector during a blood meal (FIG. 2). Within the fly, the parasite progresses through the procyclic form (PCF) and other stages while moving from the gut to the salivary glands. Finally, to complete the life cycle, the parasite is then transmitted, as the metacyclic form, to a new mammalian host in the saliva during another blood meal. The long, slender BSF and PCF stages are readily propagated in the laboratory. These two stages differ dramatically in their metabolism. For example, the PCF of *T. brucei* is able to generate ATP despite low levels of glucose using oxidative phosphorylation in a fully developed mitochondrion. By contrast, BSFs, which dwell in a glucose-rich environment, produce ATP by glycolysis, and their mitochondria do not have a conventional electron-transport chain or Krebs cycle[6].

Does the BSF of *T. brucei* synthesize FAs?

For almost 30 years, the accepted view was that the BSFs of *T. brucei* do not synthesize FAs. In 1971 it was reported that BSF trypanosomes, even when incubated in defatted medium, could not incorporate radiolabel from exogenous [^{14}C]acetate into FAs[7]. As acetate will label FAs in most prokaryotic and eukaryotic cell types under similar conditions, this experiment was interpreted as evidence that the BSFs of trypanosomes were incapable of FA synthesis. Although these parasites efficiently salvage exogenous radiolabelled FAs[8,9], free FAs are not abundant in serum[10]. Further studies led to the conclusion that receptor-mediated lipoprotein (low-density lipoprotein and high-density lipoprotein) uptake was the source of most *T. brucei* lipids with FAs supplied in the form of cholesterol esters and phospholipids[11–14]. The fact that host blood is rich in esterified FAs reinforced the view

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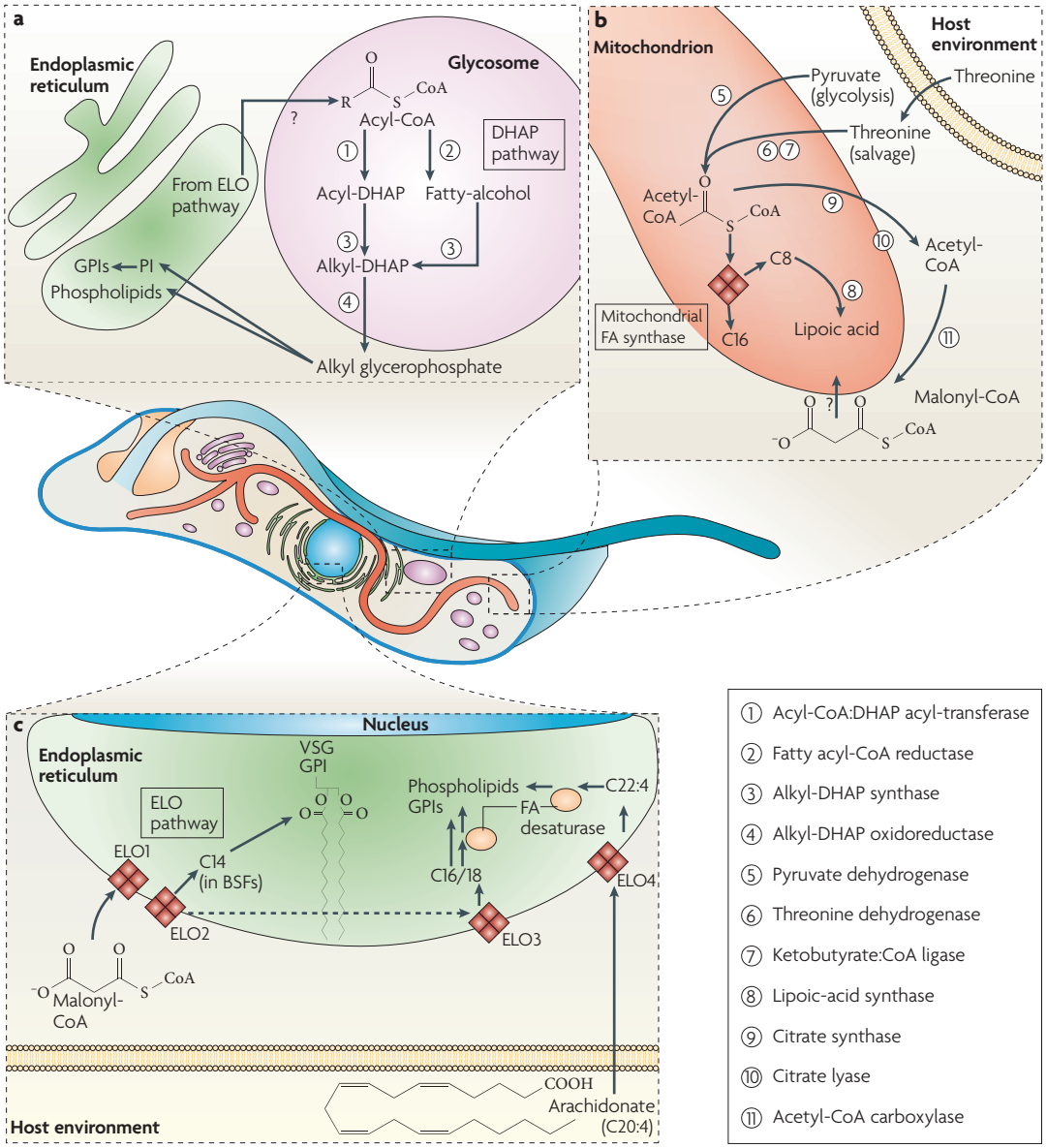

Figure 1 | ***Trypanosoma brucei* organelles and fatty-acid-related pathways. a** | The glycosomal dihydroxyacetone phosphate (DHAP) pathway uses long-chain acyl-coenzyme As (acyl-CoAs) (presumably derived from the elongase (ELO) pathway) to make fatty alcohols for ether lipid synthesis. The first step in this pathway is the synthesis of acyl-DHAP by acyl-CoA:DHAP acyl-transferase (1). Then the acyl group is displaced by the fatty alcohol in a reaction that is catalysed by alkyl-DHAP synthase (3). Finally, alkyl-DHAP oxidoreductase (4), which is associated with the cytosolic face of the glycosomal membrane[39], reduces alkyl-DHAP to alkyl-glycerophosphate. Cytoplasmic alkyl-glycerophophate then enters the endoplasmic reticulum and can be used directly for phospholipid synthesis or converted to phosphatidylinositol (PI) for glycosylphosphatidylinositol (GPI) synthesis (in *Leishmania major* and *Trypanosoma cruzi*). **b** | Acetyl-CoA, which is derived from pyruvate (from glycolysis) or threonine (salvaged from extracellular sources), is incorporated into fatty acids (FAs) by the mitochondrial FA synthase. (The four quadrants shown in the mitochondrial FA synthase represent the β-ketoacyl-ACP synthase (KAS), β-ketoacyl-ACP reductase (KAR), β-hydroxyacyl-ACP dehydratase (DH) and *trans*-2-enoyl-ACP reductase (ENR) enzymes as discussed in the main text.). This can be achieved either directly (assuming acetyl-CoA can serve as primer) or by conversion into malonyl-CoA. Production of malonyl-CoA by acetyl-CoA carboxylase (11) can occur in the cytoplasm, but how it gets back into the mitochondrion is not known. Additionally, it has not been established how acetyl-CoA exits the mitochondrion; either as citrate (9, 10) (as depicted in the figure and REF. 68) or as acetyl-L-carnitine[97,98]. Mitochondrial FA synthase makes products up to C16, and also makes C8 for lipoic-acid synthesis. **c** | ELO1 (the four quadrants correspond to the four activities in FIG. 3) and ELO2 use malonyl-CoA to elongate a butyryl-CoA primer to myristate (C14). Myristate can be incorporated into the variant surface glycoprotein (VSG) GPI anchor (in the bloodstream form (BSF) of *T. brucei*) or it can be elongated by ELO3 to make C16 and C18. These longer FAs are used directly in GPIs and phospholipids, or modified by FA desaturases (FADs) before their incorporation into phospholipids. ELO4 elongates arachidonate (C20:4 from extracellular sources) to C22:4, which can be elongated and desaturated further. Arachidonate and other polyunsaturated FAs (PUFAs) are incorporated into phospholipids. In *L. major* and *T. cruzi*, unsaturated FAs are also used for GPIs (TABLE 1).

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that BSF trypanosomes do not synthesize FAs *de novo*. Despite the conclusions reached from the above studies, as the PCF of the parasite is able to synthesize FAs[15], it is clear that the trypanosome genome must encode FA synthesis machinery.

The first clue that the BSF of *T. brucei* might have the ability to synthesize FAs came from studies on the variant surface glycoprotein (VSG), the parasite molecule that mediates antigenic variation[16]. The glycosylphosphatidylinositol (GPI) anchor of VSG is unusual in that both of the FA molecules are myristate (C14)[17] (see BOX 1 for an overview of FA nomenclature). Because VSGs are present in high copy numbers (10^7 molecules per cell)[16] and the BSF of trypanosomes reaches high densities in blood (exceeding 10^9 cells per ml in rodents), there is an enormous requirement for myristate. The realization that the levels of myristate in blood are too low to support myristoylation of the VSG GPI anchor at high parasitaemia led to speculation regarding alternative sources of myristate, and whether the BSF of trypanosomes, in contrast to dogma, might indeed synthesize FAs[3].

Biochemical studies soon revealed that trypanosome membrane preparations, from either the BSF or the PCF of the parasite, robustly synthesize FAs in a cell-free assay. Like other FA-synthesis systems, [^{14}C]malonyl-coenzyme A (CoA) (the two-carbon donor) and NAD(P)H (the reducing agent) were required for FA synthesis in trypanosomes[18]. Curiously, the trypanosome system was primed by the four-carbon butyryl-CoA — acetyl-CoA could not serve as a primer as it does in other systems. One remarkable finding from this study was that the FA products that are synthesized by the PCF and BSF of the parasite differed — the largest FA product synthesized by the PCF was stearate (C18); however, in the BSF, it was predominantly myristate (C14)[18]. This latter finding was consistent with the requirement for myristate in VSG GPI anchors of the BSF of trypanosomes. Interestingly, the reason why the original experiment in 1971 (REF. 7) did not reveal FA labelling by [^{14}C]acetate is that this substrate is not taken up by BSF parasites[18]. An alternative procedure for radiolabelling FAs in live BSFs, discovered later, is to add [^{14}C]threonine to the medium[19]. This compound is taken up by the trypanosome and catabolized to acetyl-CoA. Acetyl-CoA is probably carboxylated by acetyl-CoA carboxylase to form the malonyl-CoA substrate for the FA synthase (FIG. 1b).

Conventional FA synthases

With the discovery that *T. brucei* could synthesize FAs, the next objective was to identify and understand the biology of the enzyme FA synthase. 'Textbook' biology maintains that cells use either a type I or type II synthase for their bulk FA-synthesis requirements. These enzyme systems catalyse similar reactions but they differ markedly in their structure. The type I synthase, localized in the cytosol of animal and fungal cells, arranges its multiple catalytic activities in separate domains on one or two large homodimeric polypeptides[20]. By contrast, the type II FA synthase of plants and bacteria maintains each catalytic activity on an individual polypeptide[21]. Proteins in both systems are soluble and include an acyl carrier domain or protein (ACP) with a phosphopantetheine prosthetic group. The ACP shuttles the growing acyl chain or malonate, each of which is thioesterified to the prosthetic group, to the various active sites of the FA synthase. The chemical reactions catalysed by both enzyme types are analogous to those illustrated in FIG. 3 (except ACP is used as a carrier instead of CoA). Each cycle of synthesis results in the growth of the acyl chain by two carbons that are donated by a malonyl group, which has been transferred from malonyl-CoA to ACP. One cycle of two-carbon addition requires the four steps of condensation, reduction, dehydration and reduction (FIG. 3). Typical FA end products are C14 (yeast), C16 (mammals), and C18 (bacteria) molecules. The question that required investigation was whether synthesis of FAs by *T. brucei* used a type I or type II FA synthase.

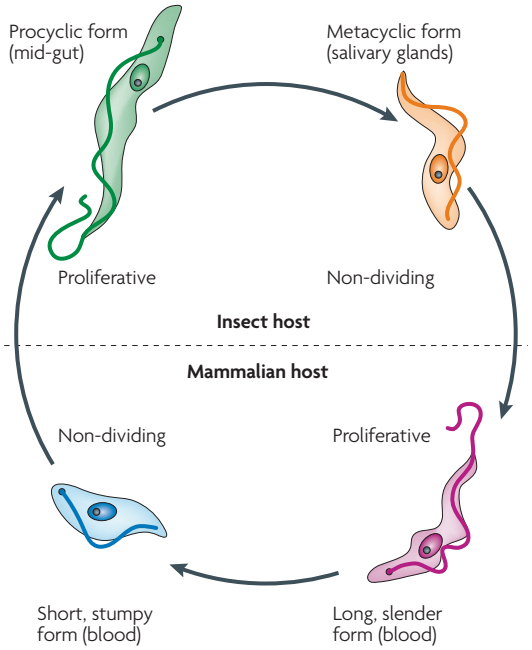

Figure 2 | **Life cycle of *Trypanosoma brucei*.** The life cycle of *T. brucei* alternates between the insect vector (the tsetse fly (*Glossina morsitans*)) and a mammalian host (such as humans, cattle, antelopes, buffaloes and lions). This figure is a simplified life cycle showing only the best-studied stages that are found in the mammalian bloodstream and the tsetse vector. In both the insect vector and the mammalian host, colonization occurs through the proliferation of rapidly dividing trypanosome forms. These forms eventually transform into resting (non-dividing) parasite cells that are pre-programmed for cellular differentiation after changes in the environment (that is, transfer from one host to the other). Each form of the parasite shown here is covered with coats composed of glycosylphosphatidylinositol (GPI)-anchored glycoproteins. Procyclin forms the coat of the procyclic form of *T. brucei*, and the coat expressed by the other three stages is composed of variant surface glycoprotein (VSG). Modified with permission from *Nature Reviews Microbiology* REF. 100 © (2006) Macmillan Publishers Ltd.

Glycosylphosphatidylinositol (GPI). A glycolipid, covalently linked to the C terminus of many eukaryotic proteins, which anchors them to the plasma membrane. Some polysaccharides, such as lipophosphoglycan (LPG) in *Leishmania* spp., are also GPI anchored.

Primer
A short, pre-existing acyl group that initiates chain growth in FA synthesis.

Phosphopantetheine
A component of CoA or a prosthetic group of ACP; during FA synthesis the growing acyl chain is thioesterified to the phosphopantetheine sulphydryl group.

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Box 1 | **Fatty-acid nomenclature**

A fatty acid (FA) is an aliphatic carboxylic acid that is usually unbranched. FAs can be grouped by chain length where short, medium, long, and very long chain FAs are C4–C6, C8–C12, C14–C18 and C20 or longer, in corresponding order. Because they are synthesized in two-carbon steps, most naturally occurring FAs have an even number of carbons. FAs are referred to by common (myristic acid or myristate), systematic (tetradecanoic acid or tetradecanoate) and shorthand (14:0 or C14) names, where the number after the colon indicates the number of double bonds (see table below). FAs with one double bond are monounsaturated and those with more are polyunsaturated (PUFA). PUFAs usually contain non-conjugated *cis* double bonds and chemists count these from the carboxyl end using a delta symbol (for example, 20:4$\Delta^{5,8,11,14}$). However, because the chain is lengthened from the carboxyl end, biochemists count carbons from the invariant methyl (or omega) end to indicate the position of the double bonds. For example, the '6' in 20:4n-6 (or 20:4ω–6 in old literature) is the carbon position of the first double bond from the methyl end. The remaining double bonds are then deduced as they are typically spaced three carbons apart (20–6=14, 14–3=11, 11–3=8 and 8–3=5; thus 20:4$\Delta^{5,8,11,14}$). The table gives some systematic and common names of FAs relevant to trypanosomatids.

Systematic name (common name)	Shorthand designation
Butanoic acid (butyrate)	4:0
Octanoic acid	8:0
Tetradecanoic acid (myristate)	14:0
Hexadecanoic acid (palmitate)	16:0
Octadecanoic acid (stearate)	18:0
9-Octadecenoic acid (oleate)	18:1n-9
9,12-Octadecadienoic acid (linoleate)	18:2n-6
6,9,12-Octadecatrienoic acid (γ-linolenate)	18:3n-6
9,12,15-Octadecatrienoic acid (α-linolenate)	18:3n-3
5,8,11,14-Eicosatetraenoic acid (arachidonate)	20:4n-6
7,10,13,16-Docosatetraenoic acid	22:4n-6
4,7,10,13,16-Docosapentaenoic acid	22:5n-6
4,7,10,13,16,19-Docosahexaenoic acid	22:6n-3

FA synthases are encoded by the *T. brucei* genome

It was easy to rule out a type I synthase because the *T. brucei* genome (see the GeneDB: *Trypanosoma brucei* genome web page) does not encode this enzyme. However, the genome does encode components of a type II synthase and this enzyme system was initially assumed to be responsible for the observed, cell-free FA synthesis. However, experimental analysis of the properties of the FA synthase of *T. brucei* cast some doubt on this conclusion and raised the possibility that trypanosome FA synthesis is neither type I nor type II (REF. 2). For example, *T. brucei* FA synthesis activity was found to be membrane-associated whereas type II proteins were found to be soluble[18]. Most notably, RNA interference (RNAi) knockdown of ACP, a key player in type II systems, did not affect trypanosome FA synthesis[2].

In fact, research has revealed that trypanosomes make the bulk of their FAs by a new and unexpected mechanism involving endoplasmic reticulum (ER)-based elongases (ELOs) — the type II FA-synthase system has a more specialized role in *T. brucei*. The biology of the FA-elongation mechanism is described below.

HXXH amino-acid motif
A sequence, composed of two histidines (H) and two non-conserved amino acids (X), that is found in FA ELOs.

T. brucei ELOs synthesize FAs *de novo*

Genes involved in FA elongation were first discovered in yeast in 1996 (REF. 22), although the chain-elongating activity of the pathway was initially documented in the 1970s. The ELO pathway uses biochemical reactions similar to those used in the type I and II FA-synthase pathways, except the fatty acyl and malonyl groups are esterified to CoA rather than ACP. Also, the enzymes in the pathway are integral membrane proteins in the endoplasmic reticulum[23]. The ELOs, which have 5 or 7 predicted transmembrane helices and an HXXH amino-acid motif, are required for the initial condensation step of the four-reaction cycle (FIG. 3), and contain the molecular rulers that determine chain-length specificity. Conventional ELOs make long-chain FAs even longer. The yeast ELO system acts on saturated acyl chains with ELO1 extending C14 to C18, ELO2 elongating C18 to C22, and ELO3 extending C22 to C26 (REF. 24). Two yeast ketoacyl-CoA reductases[23,25] (known as YBR159W and AYR1 in *Saccharomyces cerevisiae*) and a single enoyl-CoA reductase (EnCR shared by the ELOs; known as TSC13 in *S. cerevisiae*) have been identified[23]. Mouse and human genomes each encode at least six ELOs[26], half of which are predicted to be involved in the extension of saturated and monounsaturated very-long-chain FAs and the other half are thought to extend polyunsaturated FAs (PUFAs) (BOX 1). Plants have similar elongating activity but their FA ELO enzymes (FAEs) are not homologous to the ELOs that are expressed by mammals or yeast. Plant FAEs have one or two transmembrane helices and a conserved active-site cysteine that is part of a catalytic triad not found in ELOs[27,28]. Surprisingly, however, plant FAEs can complement yeast ELO mutants[29]. It is interesting to note that the plant *Arabidopsis thaliana* genome also encodes *ELO* genes of unknown function[29].

So far, four ELOs (encoded by *ELO1–4*) and an EnCR that are homologous to their animal counterparts have been found in *T. brucei*[2]. The four ELOs diverge in a phylogenetic tree, with *ELO4* (encoded on chromosome 5), branching away from *ELO1–3* (linked in tandem on chromosome 7) (FIG. 4; see legend for accession numbers and links to the National Center for Biotechnology Information database). The first indication that ELOs were involved in FA production was the discovery that inhibiting ELO1 expression (by RNAi) virtually eliminated cell-free FA synthesis by PCF membranes. Subsequent studies, which were aimed at examining the chain-length specificities of the ELOs, used cell-free assays of membranes that were extracted from the BSF of mutant *ELO* trypanosomes. These studies revealed that ELO1–3 together account for synthesis of saturated FAs up to a chain length of C18 (REF. 2) (FIG. 1). ELO1 converts C4 to C10, ELO2 extends the chain length from C10 to myristate (C14), and ELO3 extends myristate to C18. There is some overlap in ELO specificity; for example, ELO1 can extend a C10 primer to C12, albeit with low activity. One remarkable feature of the trypanosome ELO system is that it uses short-chain acyl-CoA primers that can hardly be considered 'fatty.' This characteristic distinguishes the ELOs of trypanosomes from conventional ELOs and allows the former to synthesize FAs

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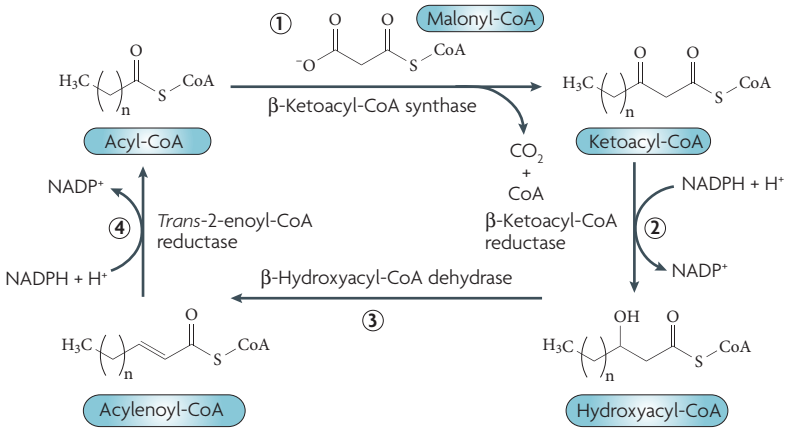

Figure 3 | **Enzymology of the elongase (ELO) pathway.** In step 1, β-ketoacyl-coenzyme A (CoA) synthase (ELO) condenses a long-chain acyl-CoA (where n is an even number) with two carbons from malonyl-CoA to form β-ketoacyl-CoA. In step 2, β-ketoacyl-CoA reductase reduces β-ketoacyl-CoA to β-hydroxyacyl-CoA, which, in turn, is dehydrated in step 3 by β-hydroxyacyl-CoA dehydrase. The resulting acylenoyl-CoA is reduced by *trans*-2-enoyl-CoA reductase in step 4 to produce a saturated acyl-CoA chain. NADPH is required for steps 2 and 4. Type I and type II fatty-acid synthases use similar chemistry, but their growing acyl chain and malonate are linked to acyl carrier protein (ACP) and not to CoA. Reproduced with permission from *Cell* REF. 2 © (2006) Elsevier Ltd.

de novo. As is discussed below, a selective downregulation of ELO3 synthesis provides an explanation for how myristate is the predominant FA product occuring in the BSF of trypanosomes.

T. cruzi and *Leishmania major* encode other ELOs

Promastigotes of *Leishmania* spp. can grow in chemically defined medium that lacks lipid moities[30]. Therefore, these organisms possess the capacity to synthesize FAs *de novo*. Indeed, similar to *T. brucei*, the genome of *L. major* only encodes a type II FA synthase[3]. This is also the case for *T. cruzi*. It is likely that *L. major* and *T. cruzi* use ELOs for FA synthesis because the conditions required for cell-free FA synthesis (including the utilization of butyryl as a primer) resemble those required for *T. brucei*[2]. Furthermore, all three parasites produce C18 — both saturated and unsaturated — in the cell-free assay, which raises the possibility that *L. major* and *T. cruzi* express ELOs with specificities that are similar to ELO1–3 of *T. brucei*[2].

Both parasite genomes encode *T. brucei* ELO1–4 (TbELO1–4) orthologues (annotated based on Jaccard clustering on the GeneDB web page) as well as additional ELO-like open reading frames (ORFs). The *L. major* genome encodes 14 predicted ELO ORFs, 5 of which are TbELO1–4 orthologues (the fifth ELO ORF is orthologous to TbELO3). *T. cruzi* encodes five predicted ELO ORFs, four of which are TbELO1–4 orthologues. A phylogenetic tree shows that, in addition to the *L. major* and *T. cruzi* TbELO1–3 orthologues (highlighted in bold in FIG. 4), the fifth *T. cruzi* ELO ORF and the eight extra *L. major* ELO ORFs also group with TbELO1–3 (FIG. 4; TcD, LmA2–3 and LmD1–6). This clustering indicates that these additional ELO enzymes are involved in elongating saturated FA chains. Saturated FA chain ELOs commonly diverge from ELOs that extend unsaturated FA chains[27]. As described in the next section, the two ELOs encoded by *L. major* and the single ELO of *T. cruzi* that cluster with TbELO4 function in the synthesis of unsaturated FAs.

Synthesis of PUFAs

T. brucei, *T. cruzi* and *L. major* lipids contain PUFAs that are synthesized by the action of ELOs and desaturases. ELO4, expressed by *T. brucei*, is an example of an ELO that is specific for PUFAs — it extends arachidonate (C20:4) by two carbon atoms[2]. Although arachidonate is available for salvage from the host serum, other PUFAs must be synthesized (FIG. 1c).

In all three trypanosomatids, the stearate that is produced by the ELO pathway (in *T. brucei* by ELO1–3) can be desaturated to make C18:1 and C18:2 molecules[31,32]. However, the trypanosomatids can produce much longer PUFAs: up to 22 carbons with as many as 6 double bonds[10,33–35]. Recently, Tripodi and collaborators expressed candidate PUFA desaturases from the three trypanosomatids in yeast and characterized their substrate specificities[32] (BOX 2). *L. major* has the capacity to convert C18:2 to arachidonate and to even larger and more unsaturated PUFAs (up to 22:6); so, this organism can synthesize all of the PUFAs it requires from stearate. Analysis reveals that *T. brucei* and *T. cruzi* have apparently lost the desaturase proteins that are required for further processing of C18:2. However, these parasites have retained enzymes that act downstream in the PUFA-synthesis pathway[32], which allows them to synthesize larger PUFAs from arachidonate or other unsaturated FAs that have been salvaged from the host.

In addition to desaturases, the synthesis of larger PUFAs requires ELOs. Tripodi and colleagues predicted the presence of two PUFA ELO enyzmes in *L. major* and, at most, one PUFA ELO in *T. brucei* and *T. cruzi*[32]. Indeed, biochemical studies confirmed *T. brucei* ELO4 as the sole ELO involved in the synthesis of PUFAs[2]. An ELO open reading frame (ORF) from both *T. cruzi* and *L. major* branches with TbELO4 in a phylogenetic tree (FIG. 4, TcE and LmE1). A second ELO ORF encoded by *L. major* (FIG. 4, LmE2) branches near the PUFA ELO cluster and is distant from saturated chain ELOs. This latter enzyme has recently been confirmed as the PUFA ELO that is responsible for extension of C18:3 and C18:4 (REF. 36).

Acyl and alkyl chains of trypanosomatid GPIs

Does the biology of *T. cruzi* and *L. major* require additional ELOs? It is likely that these parasites use these extra enzymes to build the long-chain acyl and alkyl components, both saturated and unsaturated, of their abundant surface GPI anchors and glycoconjugates (TABLE 1). In the case of *T. brucei*, analysis of the FA components of GPI anchors and phospholipids revealed that there were no saturated FAs longer than C18 (REFS 33,37) (TABLE 1), which indicates that ELO1–3 were sufficient for their synthesis. As mentioned above, *T. cruzi* and *L. major* have one and eight additional ELO ORFs,

Epimastigote, promastigote and trypomastigote
Life-cycle stages of trypanosomatid protozoans that differ morphologically in the positioning of the flagellum.

Jaccard clustering
An algorithm for grouping highly related proteins.

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Ceramide
A molecule composed of sphingosine and an FA that forms the lipid moiety of some GPI anchors and the phospholipid sphingomyelin.

respectively, which group with TbELO1–3. These enzymes are probably involved in the synthesis of the very-long-chain saturated acyl and alkyl groups that are listed in TABLE 1. In *L. major*, the GPI anchor of the promastigote surface protease (PSP) contains a C24 or C26 alkyl chain, and lipophosphoglycan (LPG) contains a C22, C24 or C26 alkyl chain[38]. Similarly, the GPI anchor of metacyclic mucin and epimastigote glycoinositolphospholipid (GIPL) of *T. cruzi*, are both based on ceramide molecules that contain a C16 or C24 acyl chain[38].

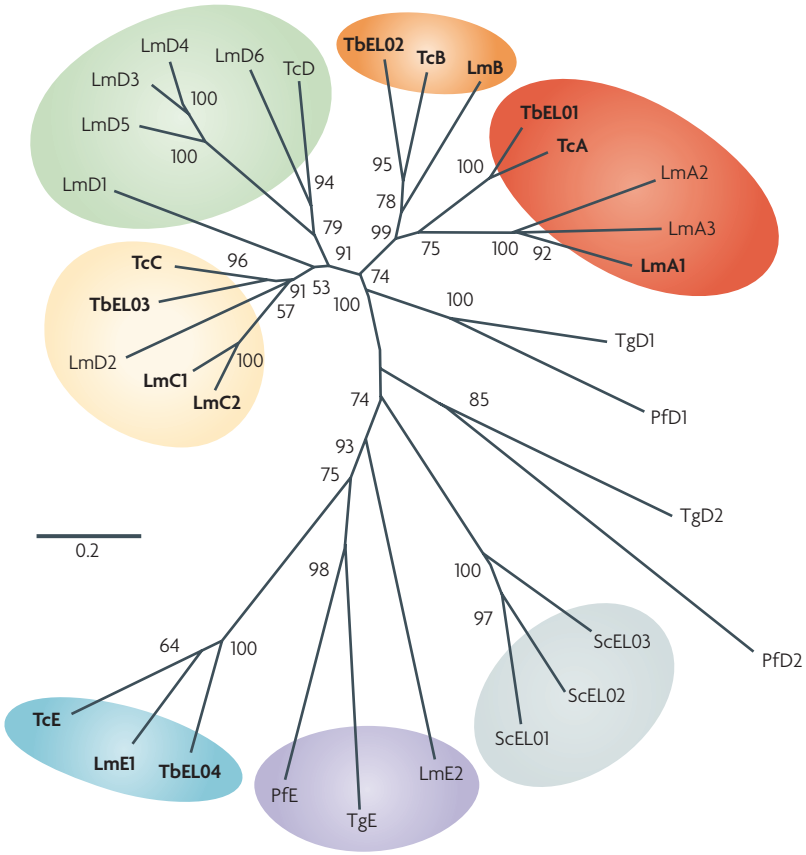

Figure 4 | **Phylogenetic tree of trypanosomatid, apicomplexan and yeast elongases (ELOs).** Protein alignments were made with CLUSTALW and an unrooted neighbour-joining tree was obtained using MEGA[99] version 3.1. Protein distances (scale bar) were calculated by Poisson correction. The numbers on the branches (bootstrap values) indicate the percentage of times the partition of the proteins into the two sets that are separated by that branch occurred among the trees in a bootstrap analysis (500 iterations). Trypanosomatid GeneDB-annotated orthologues are highlighted in bold within colour groupings: TbELO1 and homologues (red), TbELO2 and orthologues (orange), TbELO3 and homologues (yellow), TbELO1–3 homologues (green), TbELO4 and orthologues (blue), other polyunsaturated fatty-acid ELOs (lavendar), and yeast ELOs (grey). A similar tree was obtained independently elsewhere[36]. Note the trypanosomatid ELO1–3 gene loci are synteneous and include conserved upstream and downstream genes[36]. Accession numbers in the National Center for Biotechnology Information (NCBI; except for *Trypanosoma gondii*) are listed. *T. brucei* TbELO1–4 (XP_824876, XP_824877, XP_824878 and XP_824041, respectively). *T. cruzi* TcA–E (XP_813972, XP_813971, XP_813970, XP_809644 and XP_808770, respectively). Sister alleles are not shown. *Leishmania major* LmA1–A3 (CAJ02963, CAJ02967, and CAJ02975), LmB (CAJ02982), LmC1–C2 (CAJ02986, CAJ03003), LmD1–D6 (CAJ03006, CAJ03013, CAJ03016, CAJ03023, CAJ03028, CAJ03035) and LmE1–E2 (CAJ02037, CAJ08636). *Plasmodium falciparum* PfD1 (NP_703294), PfD2 (NP_704739), and PfE (XP_966049). *T. gondii* TgD1 (20.m00392), TgD2 (52.m01617), and TgE (49.m03288). *Saccharomyces cerevisiae* ScELO1–3 (NP_012339, NP_009963, and NP_013476, respectively).

The ELO system is also implicated in the synthesis of the carbon-chain components of fatty alcohols that are ether-linked to the glycerol of GPIs and phospholipids. The fatty alcohols are formed by the reduction of long-chain acyl-CoAs. This reaction, and the initial steps of ether lipid synthesis that involve the dihydroxyacetone phosphate (DHAP) pathway, have been localized to the glycosome[39–42] (FIG. 1a). The pathway requires transport of acyl-CoA from its site of synthesis in the ER to the glycosome, and the subsequent return of alkyl-glycerophosphate to the ER, where the molecule is incorporated into phospholipids (with alkyl groups) and, via phosphatidylinositol, into GPIs[43].

The GPI molecules that are expressed by parasites, including some of those listed in TABLE 1, are involved in the triggering of host innate immunity, which is the initial line of defence against the invading parasite[44–47]. Ultimately these glycoconjugates also contribute to the development of acquired immunity. GPI molecules mediate this effect by binding to one of a family of Toll-like receptors (TLRs) on the surface of host macrophages, which initiates a signalling cascade that culminates in the production of pro-inflammatory cytokines, including IL-12 and tumour-necrosis factor (TNF). Because of their role in the development of the immune response, there has been intense interest in these molecules and many of their structures have been determined. As indicated in TABLE 1, trypanosomatid GPIs have various lipid structures, including diacylglycerol, alkylacylglycerol, monoalkylglycerol and ceramide. Fatty acyl chains can be saturated or unsaturated. In addition, the inositol ring can be acylated with an FA. Recent studies have revealed that the GPI of *L. major* LPG and the mucin GPI anchor of *T. cruzi* trypomastigotes activate TLR2 (REFS 47,48); and the ceramide-containing GIPLs of *T. cruzi* epimastigotes activate TLR4 (REFS 49,50).

It is now clear that the FA components of GPI molecules, including the chain length and degree of saturation of the acyl and alkyl chains, are important determinants in the specificity of binding to TLRs and, consequently, their biological activity[51]. A striking example of the effect of the structure of GPI acyl/alkyl groups on pro-inflammatory activity is the mucin of *T. cruzi*[52]. A comparison of the structures of mucin GPIs shows that the anchor of the BSF trypomastigote contains a C16 alkyl group and either a C16:0, C18:1 or C18:2 acyl group linked to glycerol; by contrast, the GPI anchor of epimastigote mucin contains saturated C16 alkyl and acyl chains linked to glycerol[52]. There are only minor differences in their glycan structures. Remarkably, however, the BSF trypomastigote, with its unsaturated acyl groups, has potent pro-inflammatory activity, similar to that observed with bacterial lipopolysaccharide when assayed at comparable concentrations. By contrast, the epimastigote- and metacyclic-trypomastigote-derived GPIs, which have saturated acyl and alkyl groups, are inactive. Therefore, it has been proposed that *T. cruzi* parasites could remodel the lipid moiety of GPI molecules in the infective stage so as to stimulate innate

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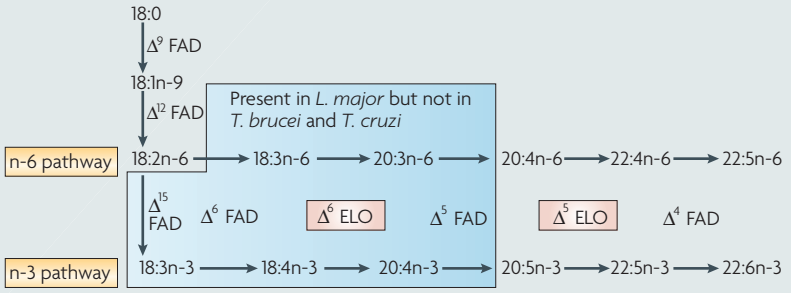
Box 2 | **Polyunsaturated fatty-acid synthesis**

On the basis of the characterization of fatty acid (FA) desaturase (FAD) specificity in *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major*, Tripodi *et al.* have proposed a pathway for polyunsaturated FA (PUFA) synthesis[32] (see BOX 1 for FA nomenclature). The pathway, shown below, begins with stearate (18:0), which, in *T. brucei*, is formed by ELO1–3 or is salvaged from the host environment. Initially, the stearate is desaturated by a Δ^9 FAD (stearoyl-coenzyme A (CoA) desaturase), which produces 18:1n-9 (oleic acid). This molecule is further desaturated by a Δ^{12} FAD to form 18:2n-6 (REFS 31, 32). All three trypanosomatids can catalyse these first two reactions; however, only *L. major* can catalyse the next steps (shaded in the figure) in which PUFA synthesis splits into the n-6 and n-3 pathways. The two pathways, n-6 and n-3, differ in their starting substrates — 18:2n-6 and 18:3n-3 — but share elongases (ELOs) and desaturases. So, *Leishmania* spp. have three additional desaturases that are absent in *T. brucei* and *T. cruzi*[32]. The first is a Δ^{15} FAD that converts 18:2n-6 to 18:3n-3 (α-linolenate). The other two desaturases (Δ^6 and Δ^5 FADs) along with an ELO (Δ^6 ELO), are involved in the synthesis of arachidonate (20:4n-6), as well as 20:5n-3, as shown in the figure[32]. Presumably, in *T. brucei* and *T. cruzi*, PUFA precursors, such as arachidonate, are taken up from the host for n-3 =and n-6 PUFA synthesis. TbELO4 (a Δ^5 ELO) converts arachidonate to 22:4n-6 (REF. 2) (as well as 20:5n-3 to 22:5n-3 (REF. 32)). *L. major* and *T. cruzi* TbELO4 orthologues (FIG. 3) catalyse the same reaction[36]. The latter product(s) is finally converted by a Δ^4 FAD to give 22:5n-6 (and 22:6n-3)[32].



immunity, thereby curbing parasite load and allowing host survival and parasite transmission[51]. As discussed below, the regulation of the ELO pathway is probably responsible for the production of different FAs at each stage of the parasite life cycle.

Regulation of the ELO pathway

The modular nature of the ELO pathway, with the varying substrate specificities of the ELO enzymes, allows the parasite to control the end product of FA synthesis, depending on the needs of the life-cycle stage and the available nutrients. Indeed, the entire ELO pathway in cultured *T. brucei* cells is upregulated under low-lipid conditions[2]. In addition, selective upregulation or downregulation of *ELO* gene expression alters the chain lengths of the FAs that are produced. The most prominent example is the previously discussed downregulation of ELO3 in the BSF of trypanosomes, which results in the production of myristate. This downregulation is reversible, in that ELO3 activity was increased when cells were transferred from blood to medium where C16 and C18 FA molecules were limiting[53]. In fact, experiments that were performed over 30 years ago gave the first indication of FA-synthesis regulation when it was observed that incorporation of radiolabel from glucose into FA molecules in the BSF of *T. lewisi* (a parasite of rats) was reduced by 50% when C16 was added to the medium[7]. The myristate requirement for VSG production by the *T. brucei* BSF, the need for longer *de novo* synthesized FAs in membrane phospholipid biosynthesis, and the nutrients available in the environment, are all factors that have to be balanced by the parasite when regulating the ELO pathway. The mechanisms that are used to sense the levels of exogenous FA molecules and control ELO activity are not yet known. Nevertheless, it is clear that the ELO pathway contributes to the special biology of these parasites.

Regulation of the ELO pathway (as well as the desaturases) in trypanosomatids could also influence the FA composition of membrane phospholipids. For example, membrane lipid composition is altered when the parasite moves between the different temperatures that exist in the insect vector and the mammalian host — changes in lipid composition are needed to adjust for the effects of temperature on membrane fluidity. In *T. cruzi*, a temperature rise resulted in an increase in the ratio of saturated to unsaturated FAs in membrane phospholipids as well as an increase in the ratio of sterols to phospholipids[54]. The authors suggested that changes in lipid composition were due to FA exchange between the neutral lipids (triacylglycerols and steryl esters) and polar lipids, as well as downregulation of desaturase activity[54]. However, the possible use of FAs synthesized *de novo* by the ELO pathway must also be considered.

Triacylglycerols and steryl esters have been proposed to act as FA reservoirs in trypanosomatids[10,54]. It could be that, in the nutrient-rich environment of the host, the parasite stockpiles lipids by uptake, synthesis or both. However, despite these FA reserves, it seems that the ELO pathway of *T. brucei* provides an essential supply of FAs for incorporation into phospholipids. Radiolabelled FAs (synthesized *de novo*) were directly incorporated into the phospholipids of the BSF and PCF of *T. brucei*. Furthermore, inhibition of the ELO pathway in PCFs (by RNAi) resulted in a growth defect that could be reversed by supplementation of the culture medium with C18 (REF. 2). Another indication that active FA synthesis is essential, as mentioned previously, comes from the observation that the BSF of *T. brucei* upregulates ELO3 activity under conditions where C16 and C18, the predominant FAs in phospholipids, are limiting[53].

Other parasitic protozoans

FA synthases in other eukaryotic parasitic protozoans also deviate from conventional systems. In *Entamoeba histolytica* (see the TIGR Database: *Entamoeba histolytica* web page), *Giardia lamblia* (see the *Giardia*DB web site) and trichomonads, the absence of 'classical' mitochondria indicates that there is no type II synthase, and further investigation also indicates that there is no type I synthase. Indeed, analysis of the *E. histolytica*, *G. lamblia*, and *Trichomonas vaginalis* (see the TIGR Database: *Trichomonas vaginalis* web page) genome databases indicates that the genes for each of the conventional systems are not present (J. Samuelson, personal communication). So, these mucosal parasites do not have *de novo* FA-synthesis capabilities and are thought to take up lipids through host lipoproteins or bile lipid micelles[55,56]. However, they are capable of

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elongating and desaturating long-chain FAs, and they can assemble GPI anchors[55]. The *E. histolytica* genome database revealed eight candidate FA *ELO* genes. These ELOs are not homologous to the animal ELOs; rather, they seem similar to plant FAEs in that they are predicted to contain two transmembrane domains each. It is worth noting that the β-ketoacyl-synthases of type I FA synthase, type II FA synthase, plant FAEs and polyketide synthases (but not ELOs) share a common ancestor[29,57].

Among the apicomplexan parasites, *Plasmodium* spp. and *Toxoplasma gondii* (see the TIGR Database: *Toxoplasma gondii* web page) have a type II pathway that is essential, and is therefore considered a potential drug target[58–60]. In both parasites, this pathway resides in the apicoplast and its function includes the production of octanoate for lipoic-acid synthesis[59,61,62]. In *T. gondii*, the type II pathway probably contributes little to total cellular FA production[59]. In contrast to *Plasmodium* spp. and *T. gondii*, *Cryptosporidium parvum*, which diverged early in the apicomplexan lineage[63] and has no apicoplast, does not encode a type II synthase[64]. Both *T. gondii* and *C. parvum* have giant type I FA synthases with 21 and 26 catalytic domains, respectively[64,65]. In contrast to its cytosolic localization in *C. parvum*[66], the type I FA synthase of *T. gondii* seems to reside in the mitochondria[65]. Curiously, the type I synthase of *C. parvum* produces very-long-chain FAs using precursors (such as C16) that are present in the external medium[64]. So, the type I synthase of *T. gondii* is thought to further elongate FAs produced by the *T. gondii* type II system. Finally, analysis of the genomes of *Plasmodium* spp. and *T. gondii* indicates that they encode three ELO ORFs each. Two are distantly related to TbELO1–3, and one is probably a PUFA ELO (FIG. 4). At least one of the ELOs that is encoded by *T. gondii* seems to be expressed in a stage-specific manner (D. Roos, personal communication). Owing to the dissimilarity between apicomplexan ELOs and ELO1–3 of *T. brucei*, it is uncertain whether the ELO pathway in apicomplexans is capable of *de novo* FA synthesis.

Mitochondrial type II FA synthesis

As discussed above, the *T. brucei* genome also encodes components of a conventional type II FA synthase that have been localized to the single mitochondrion of the parasite[67] (FIG. 1b). These components include ACP, β-ketoacyl-ACP synthase (KAS), β-ketoacyl-ACP reductase (KAR) isoforms, *trans*-2-enoyl-ACP reductase (ENR) isoforms[67], and malonyl-CoA:ACP transacylase (MAT)[68]. However, β-hydroxyacyl-ACP dehydratase (DH) was not identified, possibly because its sequence is poorly conserved among species. Additionally, there are five predicted acetyltransferases in the *T. brucei* genome and at least one of these could link the FA primer to ACP. Orthologues of the *T. brucei* genes are present in the *T. cruzi* and *L. major* genomes[3], but, in these parasites, the mitochondrial FA-synthesis pathway has not been studied at the biochemical level.

The type II pathway contributes only a small fraction of total *T. brucei* FA synthesis[67]. Instead, the pathway

Table 1 | **Fatty acyl and alkyl groups in abundant trypanosomatid GPI anchors and glycoconjugates**

Species	Life-cycle stage	Protein or glycoconjugate	Fatty groups*	Acyl or alkyl chain
Protein GPI anchors				
T. brucei	PCF	Procyclin‡	C18	Acyl chain at sn-1§
			C16	Acyl chain on inositol
	BSF	VSG	C14 exclusively	Acyl chains at sn-1 and sn-2
T. cruzi	Metacyclic	Mucin	C16 or C24	Acyl chain in ceramide
	Epimastigote	Mucin	C16 exclusively	Alkyl chain at sn-1
			C16 (77%)	Acyl chain at sn-2
	Trypomastigote	Mucin	C16 exclusively	Alkyl chain at sn-1
			C16 (37%), C18:1 (31%) or C18:2 (21%)	Acyl chain at sn-2
L. major	Promastigote	PSP	C24 or C26	Alkyl chain at sn-1
			C12, C14, C16, or C18	Acyl chain at sn-2
Non-protein glycoconjugates				
T. cruzi	Epimastigote	GIPLs	C16 (14%) or C24 (61%)	Acyl chain in ceramide
L. major	Promastigote	GIPLs	C18 or C24	Alkyl chain at sn-1
			C12, C14 or C16	Acyl chain at sn-2
	Promastigote	LPG‖	C22, C24 or C26	Alkyl group at sn-1

*Fatty groups are saturated unless otherwise indicated. ‡Procyclin glycosylphosphatidylinositol (GPI) is inositol-acylated and has a monoacylglycerol. §sn refers to the stereospecific numbering system of the phospholipids where the number indicates the hydroxyl group on glycerol. The phosphate is esterified at sn-3, and, in ether lipids, the alkyl chain is linked at sn-1. ‖LPG GPI has a monoalkylglycerol. BSF, bloodstream form; GIPLs, glycoinositol-phospholipids; *L. major*, *Leishmania major*; LPG, lipophosphoglycan; PCF, procyclic form; PSP, promastigote surface protease; *T. brucei*, *Trypanosoma brucei*; VSG, variant surface glycoprotein. See REFS 38,52.

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Table 2 | **Drugs that target type II fatty-acid synthesis**

Drug	Fungal source	Targets	Year of discovery*	IC_{50} in BSF trypanosome growth	Refs
Classical inhibitors					
Cerulenin	*Cephalosporium caerulens*	KAS‡ TbELO2 TbELO3	1972	Not tested	18,88
Thiolactomycin (TLM)	*Nocardia* and *Streptomyces* spp.	KAS	1983	150 μM	18,89
Triclosan	Synthetic	ENR	1998§	13 μM	78,90
Isoniazid	Synthetic	InhA‖	1994¶	Not tested#	91
Diazaborine	Synthetic	ENR	1981	Not tested	92
Newly developed inhibitors					
Pantothenamides	Synthetic	ACP	2004**	Not tested	93
TLM analogues	Synthetic	KAS	2005	2 μM	94
Platensimycin	*Streptomyces platensis*	KAS	2006	Not tested	95
Phomallenic acid	*Phoma* spp.	KAS	2006	Not tested	96

*The year that the target was discovered. ‡Cerulenin inhibits both type I and II KAS (β-ketoacyl-ACP synthase). §Antimicrobial activity of Triclosan was discovered in 1974. ‖InhA is the ENR (*trans*-2-enoyl-ACP reductase) of *Mycobacterium tuberculosis* mycolic-acid synthesis. ¶Antimicrobial activity of Isoniazid was discovered in 1952. #Activation of isoniazid requires modification by catalase-peroxidase (not encoded by the trypanosome genome). **Antimicrobial activity of pantothenamides was discovered in 1970. BSF, bloodstream form.

seems to have a more specialized role in trypanosome metabolism. The type II synthase produces the eight-carbon FA precursor of lipoic acid, the cofactor for α-keto acid dehydrogenase complexes. This is also the case in yeast[69], human[70] and plant[71] mitochondrial FA-synthesis pathways (it should be noted that plant cells also express a type II FA synthase in their chloroplasts for the synthesis of bulk FAs[72]). The mitochondria of *T. brucei* also synthesize their own pool of longer-chain FA molecules, the longest being predominantly C16 in size[67] (FIG. 1b). This differs in size from that observed in plant cells, which synthesize FAs of up to C18 in length[73,74]; and in *Neurospora crassa*, which makes FA molecules of up to C14 (REF. 75). Genetic studies in yeast and in *N. crassa* that have probed the function of these longer-chain mitochondrial FA molecules indicate that they could be used for the synthesis of the mitochondrial phospholipid cardiolipin[76], or could be used locally to repair damaged phospholipids[77].

Inhibitors of FA synthesis

The ELO pathway is essential to the PCF[2] and BSF (S.H.L., unpublished observations) of *T. brucei*. Moreover, studies of ACP and KAS indicate that the mitochondrial pathway is also essential in *T. brucei*[67]. As such, both pathways are potential targets for the development of new drugs to treat African sleeping sickness. Several classical FA-synthesis inhibitors have already been tested. KAS inhibitors, such as cerulenin and thiolactomycin, effectively target trypanosome FA synthesis[18]. Cerulenin was shown to inhibit ELO2 and ELO3 in a cell-free assay. ELO1 was not inhibited, probably because the alkyl chain of cerulenin is too long to bind to the active site of the enzyme[2,18]. Thiolactomycin, on the other hand, seems to inhibit both the ELO and the mitochondrial pathways in cell-free assays (J.L.S., unpublished observations), and also kills cultured trypanosomes with an IC_{50} of 150 μM (REF. 18). Targeting two essential pathways would increase the susceptibility of the trypanosome to this and other drugs. Triclosan, an inhibitor of type II ENRs, kills trypanosomes at concentrations that have little effect on ELO activity[78]. The efficacy of this drug is probably due to non-specific membrane perturbation; however, its effect on mitochondrial ENR has not been assessed. The recent interest in developing new drugs that target bacterial type II FA synthesis has the potential to provide a whole new array of inhibitors that could be effective against trypanosome mitochondrial FA synthesis (TABLE 2).

The ELO pathway in the light of evolution

The question of the origin of the trypanosome ELO enzymes is closely linked to the question of trypanosomatid evolution. The latter has been difficult to answer, in part because trypanosome nuclear genomes harbour genes that seem to be sourced from plastids and bacteria in addition to those found only in eukaryotes (such as the ELO enzymes). Parasitic trypanosomatids are descended from free-living bodonid ancestors, and their parasitism is thought to have evolved first in blood-sucking insects[79]. Multiple lateral-gene-transfer events from hosts (including plants or, in the case of bodonids, from bacterial prey) have been suggested as the source of these genes. The alternative theory, in which genes were acquired from plastids that were subsequently lost[79,80], is not consistent with recent genome analyses that have found no indication of the gene signatures that would suggest plastid symbiosis in the past[81].

Trypanosomatids, along with euglenoid algae, are part of the phylum Euglenozoa[82]. Some euglenoids are photosynthetic and acquired their chloroplasts independently from higher plants[83,84]. One photosynthetic euglenoid, *Euglena gracilis*, has numerous FA synthases. These include: a soluble, cytosolic, type-I-like high-mass FA synthase complex that uses a flavin cofactor[85]; a plastid-localized type II synthase[83]; and a mitochondrial type II system[85]. *E. gracilis* also possesses FA-elongation and FA-desaturation activities that produce C22:5n-6 and C22:6n-3 entities[86] — the same end products that are produced by trypanosomatid PUFA synthesis. We can speculate that the genes encoding type-I-like FA synthase were lost from the ancestor of parasitic trypanosomatids as it diverged from *Euglena*, became parasitic, acclimatized to nutrient-rich, but hostile, host environments, and streamlined its genome[79]. Before or during the early stages of this process, it is possible that an ancestral *ELO* gene duplicated and diverged into the paralogues that are currently encoded on the parasite genome. Understanding which FA-synthesis pathways and which ELO enzymes are expressed by bodonids would help in understanding how the trypanosomatid ELO pathway evolved to its current level of functionality.

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Concluding remarks

The ELO mechanism is not simply an example of a peculiar metabolic pathway in an ancient eukaryote. On the contrary, it seems to have evolved to become ideally suited for the lifestyle of the parasitic trypanosomatids. In moving between different compartments in the vector and the host, the modular ELO system allows synthesis of different FA products, depending on the needs of the parasite and the availability of salvageable FAs from the environment. Indeed, the production of myristate by the BSF of the trypanosome, which is required for GPI-anchor synthesis, is achieved by selective downregulation of ELO3 activity. We can further speculate that when the BSF invades the cerebrospinal fluid, an environment where the FA concentration is much lower than in blood, the entire pathway is upregulated to produce more C16 and C18 for phospholipid synthesis. It would be interesting to investigate the mechanisms that determine how trypanosomatids sense the need for synthesis of certain FAs and, when necessary, alter the expression or activity of particular ELOs. The 14 ELOs that are encoded by *L. major* are probably regulated to produce the various long-chain acyl and alkyl groups that are needed for the GPI anchors and glycoconjugates of the different life-cycle stages[87]. However, the long-chain specificity of the ELOs that are expressed by *L. major* and *T. cruzi* awaits confirmation. Given its seemingly singular evolution in the trypanosomatid parasites, perhaps it is not surprising that the ELO pathway is essential for the viability of *T. brucei*, and probably also for *L. major* and *T. cruzi*. Finally, as these parasites inflict terrible disease on a global scale, it is critical to investigate whether the trypanosomatid ELO pathway represents a useful chemotherapeutic target against these diseases.

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Competing interests statement

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