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Acylation in trypanosomatids: an essential process and potential drug target

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Abstract

Fatty acylation—the addition of fatty acid moieties such as myristate and palmitate to proteins—is essential for the survival, growth, and infectivity of the trypanosomatids: *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania*. Myristoylation and palmitoylation are critical for parasite growth, targeting and localization, and the intrinsic function of some proteins. The trypanosomatids possess a single N-myristoyltransferase (NMT) and multiple palmitoyl acyltransferases, and these enzymes and their cellular targets are only now being characterized. Global inhibition of either process leads to cell death in trypanosomatids, and genetic ablation of NMT compromises virulence. Moreover, NMT inhibitors effectively cure *T. brucei* infection in rodents. Thus, protein acylation represents an attractive target for the development of trypanocidal drugs.

Keywords

myristoylation; palmitoylation; virulence; trafficking

Protein Acylation

Fatty acylation is the covalent addition of fatty acids to proteins. The most prevalent fatty acids added to proteins are myristate and palmitate, which are 14- and 16-carbon saturated fatty acids, respectively. Fatty acylation is essential for a number of important biological processes, including vesicle and membrane targeting, stabilization of protein-membrane interactions, and cell signaling [1]. Although additional roles of myristate and palmitate in transmembrane protein function beyond membrane affinity have not been established, these modifications help proteins associate with membrane microdomains or protein complexes [2]. In this review, we examine the role of myristoylation and palmitoylation in protein

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trafficking, protein localization, growth, and virulence of the three major pathogenic trypanosomatid species, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania*. Studies of acylation in these organisms are significant, not only to understand the biochemistry and cell biology of trypanosomatids, but also to understand the functions of these modifications in eukaryotic systems more broadly. We also explore the present and future efforts to exploit acylation for drug design, specifically by targeting the acyltransferases that mediate the modifications.

Specific acyl modifications

Myristoylation

N-myristoylation, which occurs on approximately 0.5% of eukaryotic proteins [3], is carried out by the enzyme N-myristoyltransferase (NMT). NMT catalyzes the transfer of myristate from myristoyl coenzyme A (CoA) to the amino terminal glycine residue after the removal of the initiating methionine (Figure 1). The general consensus sequence for N-myristoylation, Met-Gly-X-X-X-Ser/Thr-X-X, is broadly conserved, although the role of the Ser/Thr at the 6th position and effects of other amino acids are not fully understood [4, 5] (Figure 2). N-myristoylation is irreversible and generally occurs co-translationally, though post-translational myristoylation can occur when proteolytic processing generates a new N-terminal glycine [3]. NMT is cytoplasmic, though some association with the membrane fraction has been observed [6]. NMTs appear to be ubiquitous in eukaryotes and have been characterized in protozoa, nematodes, fungi, insects, plants, and mammals [7, 8]. Insects, nematodes, fungi, and protozoa possess a single, essential NMT [3]. Vertebrates and plants harbor two NMT isoforms (NMT1 and NMT2) that exhibit distinct but overlapping target protein specificities [9]. Despite this overlap, ablation of NMT1 causes embryonic lethality in both *Arabidopsis* and mice, indicating that NMT1 is essential [10, 11]. The functional role of NMT2 is unclear, as NMT2 is unable to compensate for NMT1 loss, and NMT2 ablation in *Arabidopsis* only results in a mild delay in flowering [10]. At the cellular level, NMT2 deletion leads to increased apoptosis, decreased cell proliferation, and aberrant signaling [12]. In addition to N-myristoylation, an unusual variation of myristoylation on cysteine sulfhydryls (S-myristoylation) has been observed in some trypanosomatids [13, 14]. Whether S-myristoylation is carried out by NMT or a specific S-myristoyltransferase (SMT) is unknown. Protein myristoylation mediates protein-protein interactions between membrane proteins that in turn facilitates a variety of signal transduction pathways [7]. For other proteins, myristoylation facilitates an initial transient interaction with membranes that is subsequently stabilized either by ionic interactions between clusters of polybasic amino acids on the protein and anionic phospholipid head groups, or by subsequent palmitoylation.

Palmitoylation

Palmitoylation (S-palmitoylation) is the post-translational addition of a palmitate in a thioester linkage to a cysteine sulfhydryl (Figure 1). Unlike N-myristoylation, palmitoylation can be reversible, and this “dynamic” palmitoylation affords an additional level of protein regulation [15]. Palmitoylation was thought to be less common than N-myristoylation, and relatively few palmitoylated proteins had been identified. More recently, proteomic characterization of palmitoylomes in protozoa, yeast, plants, and mammals have

identified upwards of 1000 predicted palmitoylated proteins [16-19], suggesting that palmitoylation is as prevalent as N-myristoylation. *In silico* prediction of protein palmitoylation is difficult because there is no strict consensus sequence for palmitoylation [20]. However, palmitoylation often follows myristoylation, and thus many palmitoylation sites are near myristoylation sites. Algorithms utilizing sites that follow Type I (-CXXC-pattern), Type II (-XCCX-pattern), or Type III (other) pattern (**Figure 2**) have been developed using matrix mutation (MaM) in order to predict palmitoylated cysteines [21].

Palmitoylation is largely mediated by palmitoyl acyltransferases (PATs) (**Figure 1**) [22], but palmitate can be added to proteins in two additional ways—by membrane bound O-acyltransferase [23] and by autoacylation [24]. PATs are integral membrane proteins with multiple membrane-spanning domains and active sites facing the cytoplasm. Most PATs are found in the Golgi and ER; however, some localize to the plasma membrane, endosomes, and other unique cellular structures [25, 26]. PATs were first identified in yeast when two proteins with a conserved Asp-His-His-Cys domain (DHHC), Akr1 and Erf2, were identified as the enzymes responsible for palmitoylation of the small GTPase, Ras2 [27-31]. Subsequently, PATs have been examined in eukaryotes including yeast, mice, plants, protozoans, and humans [32-35]. PATs are organized in two classes—those that mediate farnesyl-dependent palmitoylation, and those that mediate myristoyl-dependent palmitoylation [36, 37]. In both cases, the addition of the isoprenyl farnesyl group or the myristoyl group is necessary for palmitoylation. When palmitoylation occurs with N-myristoylation or C-prenylation, membrane association of the substrate protein is greatly increased [38, 39]. Additionally, palmitoylation may help transmembrane proteins assume proper conformation, associate with lipid rafts, form protein complexes, and trigger additional post-translational modifications like ubiquitination [22].

Trypanosomatids

Trypanosomatids are a group of eukaryotic single-celled flagellated protozoa that can be free-living or parasites of plants, insects, and vertebrates. The three trypanosomatids that cause human disease are *Leishmania* spp., *Trypanosoma brucei*, and *Trypanosoma cruzi*, which are responsible for visceral/cutaneous leishmaniasis, African sleeping sickness, and Chagas disease, respectively. Three conspicuous structures in trypanosomatids are the kinetoplast, flagellum, and flagellar pocket. The kinetoplast is a region within the mitochondrion containing the unusual mitochondrial DNA for which the class *Kinetoplastida* is named. The flagellum is a motor and sensory organelle [40], and the flagellar pocket is a specialized invagination of the plasma membrane near the kinetoplast at the site where the flagellum emerges from the cell body [40]. The flagellar pocket serves as the site of all endocytic and exocytic trafficking. Because all flagellar membrane proteins must pass through the flagellar pocket, it serves as the platform for sorting membrane proteins to the flagellum or the cell body (pellicle) [40]. Recent work has shown that proper trafficking of proteins to the flagellar and pellicular membranes is dependent upon protein acylation.

Acylation in trypanosomatids

In the trypanosomatids, 0.75% of *L. major* proteins, 0.76% of *T. brucei* proteins, and 0.54% of *T. cruzi* proteins are predicted to be myristoylated based on bioinformatics [41]. At least 124 palmitoylated proteins have been identified in the procyclic form of *T. brucei* using the acyl biotin exchange method [16]. Although no palmitoyl proteome has been produced to date in *T. cruzi* and *Leishmania* spp., numerous palmitoylated proteins have been experimentally identified [42–44]. Like many eukaryotes, trypanosomatids possess a single NMT, several putative PATs, and multiple PATs (20, 12, and 15 DHHC-containing proteins in *L. major*, *T. brucei*, and *T. cruzi*, respectively) (Goldston et al., unpublished) [16]. Collectively, protein acylation in trypanosomatids is required for diverse processes in cell growth, protein trafficking, and protein function.

Fatty acid production in trypanosomes

Trypanosomatids have two options for obtaining fatty acids while in their vector or host—to take them up from their environment or to synthesize their own. Little is known about fatty acid uptake mechanisms in trypanosomatids, but they can readily acquire medium and long chain fatty acids from their environment [45]. However, when *T. brucei* are grown in lipid-depleted medium, fatty acid synthesis is stimulated [46, 47], indicating that synthesis is critical when the availability of specific fatty acids in the environment is insufficient. For example, the demand for myristate is particularly high in bloodstream form *T. brucei*. The surface protein coat is composed of $\sim 10^7$ variant surface glycoproteins (VSGs), each having two myristates in its glycosylphosphatidylinositol (GPI)-anchor [48, 49] (**Box 1**). There is not enough free myristate in the bloodstream [50] to support the requirements of parasites by scavenging alone [51]. Thus, fatty acid synthesis is essential to bloodstream form *T. brucei* for GPI-anchor biosynthesis. Fatty acid synthesis may also be essential in other trypanosomatids, but this has not been tested experimentally.

Trypanosomatids possess two pathways for fatty acid synthesis—an endoplasmic reticulum (ER)-localized elongase pathway for major fatty acid synthesis, and a minor mitochondrial fatty acid synthesis pathway [47, 52, 53]. Interestingly, elongase pathways in yeast and mammals are used for extension of pre-existing long chain fatty acids [54], but trypanosomatids use their elongase pathway for *de novo* synthesis of fatty acids, including myristate and palmitate [47]. The elongase pathway involves the condensation of a butyryl-CoA primer with malonyl-CoA, the 2-carbon donor that is synthesized from acetyl-CoA by acetyl-CoA carboxylase [47]. This is followed by reduction, dehydration, and reduction steps resulting in a fatty acid chain that is two carbons longer. *T. brucei* possesses four elongase (ELO) isoforms (ELO1–4), which catalyze the condensation step of the fatty acid synthesis pathway. The ELOs exhibit specificity both for fatty acyl chain-length and for saturated or unsaturated fatty acids, thus the regulation of different ELOs can control the species of fatty acids synthesized [55]. In addition to orthologues for ELO1–4, *T. cruzi* and *L. major* possess additional ELOs that likely are responsible for elongating very-long chain polyunsaturated fatty acids, which are abundant in these trypanosomatids. The ELOs that supply the protein acylation machinery are ELO2 and ELO3, which produce myristate and palmitate, respectively [47]. ELO3 is downregulated in the bloodstream form of *T. brucei*,

resulting in the production of primarily myristate, which is likely used for VSG GPI-anchor myristoylation. ELO3 may also be down-regulated in *Trypanosoma equiperdum* and bloodstream form *Trypanosoma congolense*, whose VSG GPI-anchors are also di-myristoylated [56]. It would be interesting to see if ELO3 is also downregulated in *T. congolense* procyclic forms, as the GPI-anchor of the abundant protease resistant surface glycoconjugate is myristoylated at the *sn*-2 position [57].

Acylation and growth

Given that myristate is so crucial to infection and evasion of the host immune system, it is not surprising that disruption of myristate synthesis or use of nonfunctional myristate analogs is lethal to trypanosomatids [58, 59]. Myristoylation is essential for survival of *T. brucei*, *L. major*, and *L. donovani* [58-62]. However, since NMTs facilitate only protein myristoylation, and are not involved in GPI-anchor myristoylation, the effect of NMT depletion on trypanosomatids was difficult to predict. Depletion of *T. brucei* NMT (TbNMT) by RNAi causes an endocytic defect and an accumulation of vesicles, while pharmacologic inhibition of TbNMT causes flagellar pocket enlargement [61, 63]. These defects in protein trafficking and endocytosis suggest that NMT function is essential for membrane trafficking. NMT knockdown by RNAi is incomplete, which may explain why there is a more pronounced effect on cell morphology upon pharmacologic inhibition of NMT. In mice, TbNMT-depleted parasites cannot establish an infection, and mice clear the parasites by 4 days post-infection [61, 64]. Thus, protein myristoylation is essential for cellular growth, vesicular trafficking, and survival in the mammalian host.

Overexpression of NMT is problematic as well. In *Leishmania*, LmNMT overexpression prevents promastigotes from differentiation to metacyclics and is ultimately lethal [59]. There are severe phenotypic alterations including disrupted intracellular structures and the appearance of novel lipid-rich oval structures. NMT overexpression studies have yet to be carried out in *Trypanosoma* spp, and therefore these observations may not be true of all trypanosomatids. NMTs are overexpressed in carcinomas [7], which suggests that tight control may be necessary to maintain proper cell growth and proliferation in eukaryotes generally. In addition, knockdown/knockout of acylated proteins has been used to determine the function of these proteins and their effects on virulence. Knockout of small myristoylated protein 1 (LmSMP-1) and LmSMP-2 in *L. major* causes flagellar shortening and defective motility [65], which may affect the virulence of the parasite *in vivo*. Knockdown of myristoylated TbARL or LdARL is lethal [64].

Whereas *L. major* and *T. brucei* have only one NMT [59], they have many PATs (**Table 1**). Global inhibition of palmitoylation using the palmitate analog 2-bromopalmitate is toxic to both bloodstream and procyclic form *T. brucei*, indicating that palmitoylation is essential [16]. However, RNAi knockdown of each individual TbPAT does not affect procyclic or bloodstream form parasites *in vitro* [16]. It is essential to determine if specific PATs are responsible for palmitoylation of virulence factors that may affect survival in a mammalian host. One such virulence factor is calflagin, a flagellar calcium binding protein in *T. brucei*. Knockdown of calflagin during infection in mice causes prolonged survival and complete clearance of parasites in 30% of the animals [66]. Because TbPAT7 is solely responsible for

calflagin palmitoylation, TbPAT7 may play a role in virulence. Knockdown of TbPAT7 severely curtailed virulence; parasitemia levels were never really established, and 100% of the mice cleared the infection (Goldston et al., unpublished). Similarly, other PATs that palmitoylate essential factors for growth and differentiation may be required for virulence as well.

Acylation and trafficking

Myristoylation and palmitoylation play key roles in trafficking and localization of trypanosomatid proteins (**Table 2**). Knockdown of TbNMT produces defects/delays in trafficking of molecules such as VSG, due to mislocalization of the myristoylated Arf-like small GTPase ARL1, which is important for trafficking [61, 64]. TbARL1 is localized to the cell membrane, cytoplasm, and Golgi, and LdARL requires myristoylation for targeting to the trans-Golgi network [64, 67]. Knockdown of TbNMT results in defective endocytosis, but no gross changes to ER-Golgi-lysosomal trafficking or ER-plasma membrane trafficking [61]. Although TbNMT depletion causes a relatively mild trafficking phenotype, the resulting mislocalization of TbARL1 and defects in internalizing cargo are sufficiently detrimental to the parasite to compromise its ability to thrive *in vivo*.

Most studies of the roles of myristoylation and palmitoylation in trafficking have been conducted on dually acylated proteins. One general model is that cytoplasmic myristoylation leads to protein trafficking to the Golgi. Palmitoylation, which may occur in the Golgi or further in the trafficking pathway, promotes further trafficking to various membrane domains, including the flagellar membrane. In *Leishmania*, this is exemplified by LmSMP-1, protein phosphatases with EF-hands (LmPPEF), and hydrophilic acylated surface protein B (LdHASPb), all of which require myristoylation for Golgi association and palmitoylation for flagellar localization [41, 42, 68-70]. LdHASPb is also localized to the pellicular membrane, indicating that LdHASPb may possess additional specific signals for flagellar or pellicular localization [69]. Tbcalfagin is a myristoyl/palmitoyl protein normally found in the flagellar membrane [71]. If palmitoylation is inhibited, either by mutation of the palmitoylation site or knockdown of calflagin's PAT, myristoylated Tbcalfagin localizes to the pellicular membrane [66]. In contrast, the *T. cruzi* flagellar calcium-binding protein (TcFCaBP), which is also a myristoyl/palmitoyl dually-acylated protein, is cytoplasmic if monoacylated with myristate, suggesting species-specific differences in trafficking pathways [72, 73]. For most dually acylated proteins, only the final cellular location is known, and the specific biosynthetic and trafficking pathways have not been elucidated. Both *T. brucei* metacaspase 4 (TbMCA4) and TbCALP1.3 are localized to the flagellum [74, 75] and very likely follow one of the trafficking pathways described above.

Some dually acylated proteins in trypanosomatids localize to the pellicular membrane, indicating that factors other than acylation play a role in targeting. *T. cruzi* phosphatidylinositol-specific phospholipase C (TcPIPLC) requires myristoylation for Golgi localization and palmitoylation for association with the pellicular membrane [76], and dually acylated *T. brucei* calpain related protein 5.5 (TbCAP5.5) is similarly localized to the pellicular membrane [44]. In contrast, the monoacylated proteins LmSMP-2 and LmSMP-4 are localized to the flagellar pocket and pellicular membrane, similar to myristoylated

Tbcalflagin lacking its palmitoyl group [65, 77]. Introduction of a palmitoylation site into LmSMP-4 promotes its association with lipid rafts containing LmSMP-1, but does not redirect it to the flagellum [77]. Therefore, while palmitoylation is required for specific membrane association for some dually acylated proteins, there exist other acylation-independent localization signals that are essential for correct protein targeting. A hypothetical targeting pathway for dually acylated proteins based in large part on published results is presented (**Figure 3**).

A number of secondary flagellar localization signals have been defined. In addition to N-terminal myristoylation and palmitoylation, a polybasic stretch of amino acids near the N-terminus is necessary for the flagellar localization of TcFCaBP [72]. Tbcalflagin also possesses a lysine-rich region near the acylation site [72], so it is possible that this polybasic region is necessary for Tbcalflagin localization as well. N-terminal cysteines also appear to carry targeting information for acylated proteins. TcPIPLC, a dually-acylated protein localized to the pellicular membrane, becomes flagellar upon mutation of cysteine 8 [76]. Although the modification does not affect the acylation state of TcPIPLC, the cysteine mutation may make the N-terminus more like that of TcFCaBP and thus favors its localization to the flagellum [76]. Likewise, TbGPI-PLC has a cluster of N-terminal cysteines that are important for flagellar localization, but a C-terminal proline is also necessary for flagellar localization [78]. The C-terminal proline-containing motif may serve as a substrate for a peptidyl prolyl isomerase, which may act to re-orient the TbGPI-PLC C-terminus so that it can interact with other factors, enabling flagellar localization [78]. Lipid rafts are necessary for the targeting of proteins such as Tbcalflagin and TcFCaBP [79]; indeed a sterol-enriched flagellar membrane composition is required for localization of Tbcalflagin to the flagellum [79]. However, other proteins such as LmSMP-1 are still targeted to the flagellum after depletion of raft components, cholesterol, and sphingolipids [68].

Trafficking pathways appear to be partially conserved among trypanosomatids. Tbcalflagin and TcFCaBP both traffic to the flagellar membrane [71, 72]. In addition, TcFCaBP is flagellar and associated with lipid rafts when expressed in *L. amazonensis*, suggesting that the LaPAT involved is conserved [72]. The palmitoylation signal for LmHASPb localization is also recognized in higher eukaryotes. LmHASPb, whose endogenous localization is the pellicular membrane, traffics to the extracellular leaflet of the plasma membrane when expressed in Chinese hamster ovary cells [42].

Acylation and protein function

Many trypanosomatid acylated proteins are themselves essential for growth and/or virulence. LmSMP-1 is essential for flagellar structure, stabilization, and motility [65, 68]. TcPIPLC is a component of the inositol phosphate/diacylglycerol pathway, and is involved in differentiation of *T. cruzi* trypomastigotes to amastigotes [80]. Knockdown of Tbcalflagin leads to prolonged survival and complete clearance of parasites in 30% of the mice [66]. Dually acylated TbCAP5.5 and TbCAP5.5V are essential to procyclic and bloodstream form *T. brucei* parasites, respectively [81]. TbCAP5.5 knockdown is associated with aberrant cytokinesis and altered cell morphology [81].

Although assumed, it has not been confirmed in all cases that proper localization of these essential acylated proteins is necessary for their function. The importance of acylation in protein function is highlighted by the virulence defects that occur when proper acylation of these proteins is prevented. TbGPI-PLC, which is palmitoylated within a cluster of cysteines at positions 269, 270, and 273 [82], cleaves VSG to release the surface coat after shock or osmotic stress and during rapid surface coat switching. In the steady state, non-acylated and acylated forms of TbGPI-PLC co-exist; however, upon activation by osmotic shock there was a shift to 100% acylated form [82]. While palmitoylation does not directly affect the enzymatic activity of TbGPI-PLC, it is required for access to its lipid substrate, which creates a regulatory mechanism whereby GPI cleavage can be controlled in part through TbGPI-PLC palmitoylation and the resulting partitioning of TbGPI-PLC into the membrane [82]. TbMCA4 provides a counter-example wherein acylation is not necessary for proper activity and virulence function. TbMCA4 is dually acylated and localized to the flagellar membrane prior to secretion [75]. TbMCA4 knockout parasites are less virulent in a mouse model, but this virulence defect is rescued by introduction of either acylated or non-acylated TbMCA4^{G2A} [75]. Although TbMCA^{G2A} is cytoplasmic, it is still secreted, suggesting that localization to the flagellum is not necessary for MCA4 secretion [75].

Acylation as a drug target

There has been much excitement recently about the use of NMT inhibitors as potential new treatments for sleeping sickness. Two anti-fungal NMT inhibitors displayed *in vitro* and *in vivo* efficacy against bloodstream form *T. brucei*, however both compounds tested had only modest efficacy against the *L. major* NMT or *L. major* promastigote cell growth [83], likely due to sequence differences between the *L. major* and *T. brucei* NMTs [59]. There is considerable sequence identity among the NMTs of different *Leishmania* spp [59], suggesting that an effective NMT inhibitor against one leishmanial NMT would be broadly effective against *Leishmania*. HsNMT2 is the closest human homolog to TbNMT, with an overall 55% identity and 69% similarity; but the conservation is higher in the residues closest to the active site, with 83% identity and 90% similarity [63].

Structural modeling of the binding cavities in the *T. brucei* and *L. major* NMT proteins suggest that NMT analogs interact with the active site via hydrophobic, hydrogen bonding, and ionic interactions with both positively and negatively charged residues [84]. The major difference in *T. brucei* and *L. major* enzymes compared to other NMTs is the presence of additional positively charged residues in the binding cavity [84]. Although the *L. donovani* enzyme lacks the extra charged residues in the binding cavity, there is no structural overlap in this region between LdNMTs and either human or other protozoan NMTs, suggesting that any inhibitors of *L. donovani* NMTs will be selective [85].

Large compound libraries have been screened for activity against trypanosomatid NMTs [63, 86]. One "hit" from the library was a pyrazole sulphonamide compound, DD85646, which mimics the N-terminus of the myristate substrate and thus acts as a competitive inhibitor of NMT by occupying the basic binding site [63]. DD85646 inhibited the proliferation of bloodstream form *T. brucei* with 200-fold selectivity over mammalian cells and effectively cured mice of *T. brucei brucei* and *T. brucei rhodesiense* infections [63].

Treatment with DD85646 resulted in an enlarged flagellar pocket, suggesting endocytic defects, a phenotype similar to that observed for TbNMT RNAi [63]. In addition, DD85646 is active against TcNMT, but is only effective against *T. cruzi* in culture at 1000x concentrations compared to *T. brucei* [87]. A screen performed on *L. donovani* identified a number of compounds that showed activity against LdNMT, with no detectable activity against human NMTs or TbNMT [85]. Interestingly, these compounds appear to bind to a region of the binding pocket distinct from other previously characterized NMT inhibitors, suggesting it may be possible to exploit multiple compound classes to target NMTs [85]. Although DD85646 is an effective NMT inhibitor against bloodstream form *T. brucei* in a mouse model, further optimization is required to enable central nervous system penetration.

Because global inhibition of palmitoylation with 2-bromopalmitate is toxic to both bloodstream and procyclic form *T. brucei* [16], palmitoylation also appears to be a viable drug target. This idea is further supported by studies in *Toxoplasma gondii* that demonstrate ablation of single PATs can have adverse effects on virulence [26, 88]. Studies about the necessity of specific PATs in *Trypanosomatids* are currently being undertaken, and suggest that at least one PAT is essential (Goldston *et al.*, unpublished). However, the multiplicity of PATs potentially complicates these studies if there is significant functional overlap between PATs.

Both PATs and NMTs are being targeted as treatment for a number of human diseases. NMT inhibitors have been identified as potential targets for cancer and infectious diseases caused by bacteria, fungi, or other protozoans [89-92]. While development of PAT inhibitors is in its infancy, a strategy using acyl-CoA analogs to inhibit the autoacylation step may generate specific, powerful inhibitors [93]. As anti-PAT compounds are developed to treat one or more human diseases, it may be possible to “piggy-back” on those efforts to identify inhibitors for homologous PATs in trypanosomatids. This “piggy back” ideology has been used for both NMTs and farnesyltransferase inhibitors [94] and has led to success for farnesyltransferase inhibitors, which were first developed in cancer therapeutics, and then tested in protozoans such as *T. brucei* and *Plasmodium* [95].

Concluding remarks and future perspectives

Trypanosomatids present a unique system to study protein acylation. From the earliest understanding of the reliance of these protozoans on myristate, to the determination of the first protozoan palmitoyl proteome, to the research on NMT inhibitors as therapeutics, trypanosomatids have emerged as valuable model organisms for studying the role of acylation in biology and disease. Myristoylation and palmitoylation research in trypanosomes has elucidated trafficking pathways to the flagellum/cilium, and the novel appreciation of the ciliary membrane as a liquid-ordered membrane environment [79]. In addition, comprehensive studies on NMTs and their inhibitors have led to a better understanding of these enzymes and how they function.

Many questions have yet to be answered in the palmitoylation and myristoylation fields alike (**Box 2**). A comprehensive understanding of flagellar protein targeting in the trypanosomatids and ciliary targeting mechanisms in other eukaryotes is needed. In addition,

we do not yet understand the role that reversible palmitoylation plays in the function of eukaryotic proteins, and thus, the study of palmitoylation/depalmitoylation cycles in trypanosomatids and other protozoans may provide insight to this form of protein regulation. Finally, inhibitors for myristoylation require further testing and optimization, but may prove to be effective drug targets against diseases caused by the trypanosomatids and other infectious diseases.

Box 1

GPI-anchor myristoylation: another form of protein myristoylation

Unlike mammalian GPI-anchors, the GPI-anchors of *T. brucei*, *T. congolense*, and *T. equiperdum* are exclusively di-myristoylated [56, 96, 97]. In addition, mono-myristoylated GPI-anchors have been detected on *Leishmania* GP63 surface metalloprotease and non-protein linked free GPI-anchored glycolipids and *T. congolense* Protease Resistant Surface molecule (PRS) [57, 98, 99]. Studies of the *T. brucei* VSG GPI-anchor show that myristates are incorporated in the ER during GPI-anchor synthesis by fatty acid remodeling, where pre-existing fatty acids are sequentially removed and replaced with myristate [100]. A second GPI myristoylation pathway, myristate exchange, occurs on mature VSG GPI anchors and is thought to be an editing mechanism to ensure full myristoylation of VSG GPI-anchors [101]. In *T. brucei*, the GPI anchor mediates access to VSG-specific forward trafficking and recycling pathways [102-105]. In addition, GPI-anchor valence appears to mediate differential targeting, where monovalent GPI-anchored complexes like transferrin receptor are retained in the flagellar pocket and divalent GPI-anchored complexes like VSG are targeted to the pellicular membrane [106, 107].

The function of GPI-anchor myristoylation is unknown. Blocking GPI-anchor myristoylation by RNAi of TbGUP1, the *sn*-2 myristoyltransferase, resulted in a mild growth phenotype and a normal level of VSG expression on the surface, though the kinetics of VSG trafficking and the composition of the mature VSG GPI-anchors were not investigated [108]. The mild phenotype was likely due to compensation by the intact myristate exchange pathway. The relatively short acyl chain of myristate offers less opportunity for hydrophobic and van der Waals interactions and thus myristate has a lower membrane affinity than have longer fatty acids. Thus, one possible function of GPI-myristoylation is to reduce VSG drag in the membrane, thereby enabling the endocytic clearance of antibody-bound VSGs by hydrodynamic flow and the avoidance of complement-mediated lysis [109, 110]. A second possible function is to increase the propensity of myristoylated GPI-anchored proteins to desorb from the plasma membrane [107, 111, 112]. This desorption could assist in the rapid shedding of the VSG coat that occurs during differentiation, as only partial myristate removal by GPI-PLC would be sufficient to facilitate VSG release from the membrane [112]. Furthermore, the release of GPI-anchored proteins could contribute to parasite survival through immunomodulatory signaling via VSG-derived antigens, including the dimyristoylglycerol moiety [113, 114].

Box 2**Outstanding questions**

Although much is known about protein acylation in eukaryotes and in trypanosomatids, questions still remain. The questions listed below are those facing the field as we enter the next phase of discovery in this exciting area of trypanosomatid biochemistry and cell biology.

- **Why so many PATs?** Yeast have 7 PATs, while trypanosomatids have a predicted 12-22. Why the large number when yeast and trypanosomatids have similar genetic and cellular complexity?
- What is the role of dynamic palmitoylation in trypanosomatids? In addition to increasing membrane affinity as a permanent acyl modification, palmitoylation may also be dynamic, mediated by cycles of palmitoylation and depalmitoylation.
- Where do the various acylation reactions occur in trypanosomatids? One appealing model is that the sub-cellular localizations of myristoyl-palmitoyl proteins are determined by (1) the presence of acyl modifications, (2) the presence of additional determinants that interact with other macromolecules, and (3) the acyl trafficking pathway, including the subcellular location of the PAT that palmitoylates the substrate protein.
- What is the identity of the myristoyltransferases governing the *sn*-1 myristoylation step in GPI fatty acid remodeling and the myristate exchange proofreading pathway? The presence of a proof-reading pathway for GPI-myristoylation suggests that the myristoyl groups play an essential role in these parasites. Identification and functional characterization of these proteins may reveal the functional role of GPI myristoylation.
- What is the basis of apparent species-specific differences in acylation trafficking **among the trypanosomatids**? Calflagin and FCaBP are localized to the flagellar membrane in *T. brucei* and *T. cruzi*, respectively and are very similar in sequence. However, while myristoyl-only calflagin localizes to the pellicular membrane, myristoyl-only FCaBP is diffusely cytoplasmic.
- What is the nature of non-acylation targeting information and how are their trafficking information integrated with the acylation-dependent targeting machinery? How is the acylation signal "read" by the trafficking machinery and what role do non-acyl targeting domains play in trafficking. What other domains contain targeting information for acylated proteins?
- Are NMTs and PATs potential drug targets in diseases caused by eukaryotic **pathogens**? Fatty acylation is essential in all eukaryotes and species-specific NMTs and PATs have been found in apicomplexans (*Toxoplasma* and *Plasmodium*) and trypanosomatids. Can the differences between protozoal and

human enzymes be successfully exploited to develop specific effective anti-trypanosomal drugs?

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Glossary

2-bromopalmitate (2-Bromohexadecanoic acid)	general palmitoylation inhibitor; also has off-target effects on fatty acid CoA ligase and other steps in fatty acid metabolism.
Acetyl-CoA carboxylase (ACC)	responsible for the synthesis of malonyl-CoA, the two carbon donor for fatty acid synthesis.
Arf-like protein 1 (ARL1)	Golgi protein involved in trafficking and expressed in bloodstream form <i>T. brucei</i> ; also encoded in <i>L. major</i> and <i>T. cruzi</i> genomes.
Calflagins	myristoylated and palmitoylated EF-hand calcium binding proteins in the flagellar membrane of <i>T. brucei</i> ; <i>T. cruzi</i> homolog is FCaBP.
CAP5.5	calpain-related cytoskeleton-associated protein in the pellicular membrane of procyclic form <i>T. brucei</i> ; essential for cell morphogenesis; homolog in bloodstream form is CAP5.5v.
CALP 1.3	calpain-like protein found at the distal tip of the flagellum in <i>T. brucei</i> .
Farnesylation	addition of an isoprenoid farnesyl group to proteins with a CaaX motif.
Fatty acylation	post-translational covalent addition of a fatty acid to a protein; usually refers to addition of a myristoyl or palmitoyl group to a protein.
Flagellar calcium-binding protein (FCaBP)	dually acylated EF-hand calcium binding proteins found in the flagellar membrane of <i>T. cruzi</i> .
Flagellar pocket	subdomain of the plasma membrane from which the flagellum emerges; site of all endocytosis and exocytosis in the trypanosome; regulates sorting of proteins destined for the flagellum, plasma membrane, or flagellar pocket itself.

Flagellum	axoneme-containing organelle responsible for cellular locomotion; also serves as a sensory structure; has a membrane highly enriched in lipid rafts.
Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC)	enzyme responsible for the cleavage of GPI-anchored proteins including VSG.
Kinetoplast	specialized region within the mitochondrion containing the interlinked network of circular mitochondrial DNA (kDNA); adjacent to the flagellar basal body.
Membrane bound O-acyltransferase (MBOATS)	multipass transmembrane enzymes that have two common active site domains—a hydrophobic region containing a conserved histidine, and a hydrophilic region containing a conserved asparagine; MBOATs acylate cholesterol/diacylglycerol, amino acids, and lysophospholipids.
Metacaspases	cysteine-dependent proteases.
Myristoylation	addition of a myristoyl group (C14:0) by covalent attachment through an amide bond; occurs on an N-terminal glycine residue; increases the protein's interaction with the membrane.
N-myristoyltransferases (NMT)	enzymes that catalyze the transfer of myristate from myristoyl-CoA to an N-terminal glycine residue.
Palmitoyl acyltransferases (PAT)	enzymes that catalyze the transfer of palmitate from palmitoyl-CoA to a cysteine residue.
Palmitoylation	reversible addition of a palmitoyl group (C16:0) by covalent attachment to a cysteine residue (S-palmitoylation or S-acylation) through a thioester bond; increases the protein's interaction with the membrane.
Pellicular membrane	surface membrane of the cell body (excludes flagellum and flagellar pocket).
Phosphatidylinositol-specific phospholipase C (PIPLC)	enzyme responsible for hydrolysis of phosphatidylinositol 4,5-bisphosphate into the second messenger molecules inositol 1,4,5-trisphosphate and diacylglycerol.
Prenylation	addition of a hydrophobic prenyl group (3-methyl-but-2-en-1-yl) to a protein; thought to act as a

Protein Phosphatases with EF-Hands (PPEF)

membrane anchor; farnesylation and geranylgeranylation are two types of prenylation.

function unknown in mammals but may correlate with stress responses and proliferation signals; in trypanosomatids, dually acylated proteins expressed in the endomembrane system but do not bind calcium (degenerate).

Small myristoylated proteins (SMPs)

highly conserved family of monoacylated or dually acylated proteins that are localized to distinct membrane domains; identified in *L. major*.

Transferrin

iron binding glycoproteins in blood plasma that reversibly bind iron to regulate levels of free iron; in trypanosomatids such as *T. brucei*, endocytosed via transferrin receptors in the flagellar pocket.

Variant surface glycoprotein (VSG)

major surface coat protein of bloodstream form *T. brucei* that mediates antigenic variation; anchored to the plasma membrane by a di-myristoylated GPI-anchor.

References

1. Dunphy JT, Linder ME. Signalling functions of protein palmitoylation. *Biochim. Biophys. Acta*. 1998; 1436:245–261. [PubMed: 9838145]
2. Resh MD. Membrane targeting of lipid modified signal transduction proteins. *Subcell. Biochem*. 2004; 37:217–232. [PubMed: 15376622]
3. Martin DD, et al. Post-translational myristoylation: Fat matters in cellular life and death. *Biochimie*. 2011; 93:18–31. [PubMed: 21056615]
4. Johnson DR, et al. Genetic and biochemical studies of protein N-myristoylation. *Annu. Rev. Biochem*. 1994; 63:869–914. [PubMed: 7979256]
5. Utsumi T, et al. Amino acid residue penultimate to the amino-terminal Gly residue strongly affects two cotranslational protein modifications, N-Myristoylation and N-Acetylation. *J. Biol. Chem*. 2001; 276:10505–10513. [PubMed: 11124252]
6. McIlhinney R, McGlone K. Immunocytochemical characterization and subcellular localization of human myristoyl-CoA: Protein N-myristoyltransferase in HeLa cells. *Exp. Cell Res*. 1996; 223:348–356. [PubMed: 8601412]
7. Wright MH, et al. Protein myristoylation in health and disease. *J. Chem. Biol*. 2010; 3:19–35. [PubMed: 19898886]
8. Giang DK, Cravatt BF. A second mammalian N-myristoyltransferase. *J. Biol. Chem*. 1998; 273:6595–6598. [PubMed: 9506952]
9. Kumar S, et al. N-myristoyltransferase in the leukocytic development processes. *Cell Tissue Res*. 2011; 345:203–211. [PubMed: 21698528]
10. Pierre M, et al. N-myristoylation regulates the SnRK1 pathway in Arabidopsis. *Plant Cell*. 2007; 19:2804–2821. [PubMed: 17827350]
11. Yang SH, et al. N-myristoyltransferase 1 is essential in early mouse development. *J. Biol. Chem*. 2005; 280:18990–18995. [PubMed: 15753093]
12. Ducker CE, et al. Discovery and characterization of inhibitors of human palmitoyl acyltransferases. *Mol. Cancer Ther*. 2006; 5:1647–1659. [PubMed: 16891450]

13. Armah DA, Mensa-Wilmot K. S-myristoylation of a glycosylphosphatidylinositol-specific phospholipase C in *Trypanosoma brucei*. *J. Biol. Chem.* 1999; 274:5931–5938. [PubMed: 10026218]
14. Armah DA, Mensa-Wilmot K. Protein S-myristoylation in *Leishmania* revealed with a heterologous reporter. *Biochem. Biophys. Res. Commun.* 1999; 256:569–572. [PubMed: 10080938]
15. Linder ME, Deschenes RJ. Palmitoylation: policing protein stability and traffic. *Nat. Rev. Mol. Cell Biol.* 2007; 8:74–84. [PubMed: 17183362]
16. Emmer BT, et al. Global analysis of protein palmitoylation in African trypanosomes. *Eukaryot. Cell.* 2011; 10:455–463. [PubMed: 21193548]
17. Roth AF, et al. Global analysis of protein palmitoylation in yeast. *Cell.* 2006; 125:1003–1013. [PubMed: 16751107]
18. Kang R, et al. Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature.* 2008; 456:904–909. [PubMed: 19092927]
19. Hemsley PA, et al. A proteomic approach identifies many novel palmitoylated proteins in *Arabidopsis*. *New Phytologist.* 2013; 197:805–814. [PubMed: 23252521]
20. Smotrys JE, Linder ME. Palmitoylation of intracellular signaling proteins: regulation and function. *Annu. Rev. Biochem.* 2004; 73:559–587. [PubMed: 15189153]
21. Ren J, et al. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Engineering Design and Selection.* 2008; 21:639–644.
22. Blaskovic S, et al. What does S-palmitoylation do to membrane proteins? *FEBS J.* 2013; 280:2766–2774. [PubMed: 23551889]
23. Matsuda S, et al. Member of the membrane-bound O-acyltransferase (MBOAT) family encodes a lysophospholipid acyltransferase with broad substrate specificity. *Genes Cells.* 2008; 13:879–888. [PubMed: 18782225]
24. Duncan JA, Gilman AG. Autoacylation of G protein α subunits. *J. Biol. Chem.* 1996; 271:23594–23600. [PubMed: 8798571]
25. Ohno Y, et al. Analysis of substrate specificity of human DHHC protein acyltransferases using a yeast expression system. *Mol. Biol. Cell.* 2012; 23:4543–4551. [PubMed: 23034182]
26. Frenel K, et al. Global analysis of apicomplexan protein S-acyl transferases reveals an enzyme essential for invasion. *Traffic.* 2013; 14:895–911. [PubMed: 23638681]
27. Lobo S, et al. Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 2002; 277:41268–41273. [PubMed: 12193598]
28. Roth AF, et al. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *J. Cell Biol.* 2002; 159:23–28. [PubMed: 12370247]
29. Zhao L, et al. Erf4p and Erf2p form an endoplasmic reticulum-associated complex involved in the plasma membrane localization of yeast Ras proteins. *J. Biol. Chem.* 2002; 277:49352–49359. [PubMed: 12379641]
30. Bartels DJ, et al. Erf2, a novel gene product that affects the localization and palmitoylation of Ras2 in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 1999; 19:6775–6787. [PubMed: 10490616]
31. Putilina T, et al. The DHHC domain: a new highly conserved cysteine-rich motif. *Mol. Cell. Biochem.* 1999; 195:219–226. [PubMed: 10395086]
32. Fukata M, et al. Identification of PSD-95 palmitoylating enzymes. *Neuron.* 2004; 44:987–996. [PubMed: 15603741]
33. Ohno Y, et al. Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochim. Biophys. Acta.* 2006; 1761:474–483. [PubMed: 16647879]
34. Greaves J, Chamberlain LH. DHHC palmitoyl transferases: substrate interactions and (patho)physiology. *Trends Biochem. Sci.* 2011; 36:245–253. [PubMed: 21388813]
35. Mitchell DA, et al. Protein palmitoylation by a family of DHHC protein S-acyltransferases. *J. Lipid Res.* 2006; 47:1118–1127. [PubMed: 16582420]

36. Varner AS, et al. A fluorescence-based high performance liquid chromatographic method for the characterization of palmitoyl acyl transferase activity. *Anal. Biochem.* 2002; 308:160–167. [PubMed: 12234477]
37. Varner A, et al. Characterization of human palmitoyl-acyl transferase activity using peptides that mimic distinct palmitoylation motifs. *Biochem. J.* 2003; 373:91–99. [PubMed: 12670300]
38. Alland L, Peseckis SM, Atherton RE, Berthiaume L, Resh MD. Dual myristoylation and palmitoylation of Src family member p59^{l^yn} affects subcellular localization. *J. Biol. Chem.* 1994; 259:16701–16705. [PubMed: 8206991]
39. Shahinian S, Silvius JR. Doubly-lipid-modified protein sequence motifs exhibit long-lived anchorage to lipid bilayer membranes. *Biochemistry.* 1995; 34:3813–3822. [PubMed: 7893678]
40. Field MC, Carrington M. The trypanosome flagellar pocket. *Nat. Rev. Microbiol.* 2009; 7:775–786. [PubMed: 19806154]
41. Mills E, et al. Kinetoplastid PPEF phosphatases: dual acylated proteins expressed in the endomembrane system of *Leishmania*. *Mol. Biochem. Parasitol.* 2007; 152:22–34. [PubMed: 17169445]
42. Denny PW, et al. Acylation-dependent protein export in *Leishmania*. *J. Biol. Chem.* 2000; 275:11017–11025. [PubMed: 10753904]
43. Corvi MM, et al. Protein palmitoylation in protozoan parasites. *Front. Biosci.* 2011; 3:1067–1079.
44. Hertz-Fowler C, et al. CAP5. 5, a life-cycle-regulated, cytoskeleton-associated protein is a member of a novel family of calpain-related proteins in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 2001; 116:25–34. [PubMed: 11463463]
45. Mellors A, Samad A. The acquisition of lipids African trypanosomes. *Parasitol. Today.* 1989; 5:239–244. [PubMed: 15463224]
46. Doering TL, et al. Trypanosome metabolism of myristate, the fatty acid required for the variant surface glycoprotein membrane anchor. *J. Biol. Chem.* 1993; 268:9215–9222. [PubMed: 8486622]
47. Lee SH, et al. Fatty acid synthesis by elongases in trypanosomes. *Cell.* 2006; 126:691–699. [PubMed: 16923389]
48. Cross G. Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology.* 1975; 71:393–417. [PubMed: 645]
49. Ferguson M, et al. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science.* 1988; 239:753–759. [PubMed: 3340856]
50. Scanu AM, Spector AA. *Biochemistry and biology of plasma lipoproteins.* Dekker. 1986
51. Paul KS, et al. Fatty acid synthesis in African trypanosomes: a solution to the myristate mystery. *Trends Parasitol.* 2001; 17:381–387. [PubMed: 11685899]
52. Stephens JL, et al. Mitochondrial fatty acid synthesis in *Trypanosoma brucei*. *J. Biol. Chem.* 2007; 282:4427–4436. [PubMed: 17166831]
53. Guler JL, et al. Mitochondrial fatty acid synthesis is required for normal mitochondrial morphology and function in *Trypanosoma brucei*. *Mol. Microbiol.* 2008; 67:1125–1142. [PubMed: 18221265]
54. Kihara A. Very long-chain fatty acids: elongation, physiology and related disorders. *J. Biochem.* 2012; 152:387–395. [PubMed: 22984005]
55. Lee SH, et al. A fatty-acid synthesis mechanism specialized for parasitism. *Nat. Rev. Microbiol.* 2007; 5:287–297. [PubMed: 17363967]
56. Lamont GS, et al. Glycosyl-sn-1, 2-dimyristylphosphatidylinositol is the membrane anchor for *Trypanosoma equiperdum* and *T.(Nannomonas) congolense* variant surface glycoproteins. *Mol. Biochem. Parasitol.* 1987; 24:131–136. [PubMed: 2957588]
57. Greganova E, et al. The protease resistant surface (PRS) glycoconjugate from *Trypanosoma congolense* has an inositol-acylated glycosylphosphatidylinositol anchor, containing a significant proportion of myristate at the s-2 position. *Mol. Biochem. Parasitol.* 2010; 171:50–54. [PubMed: 20138924]
58. Doering TL, et al. Toxicity of myristic acid analogs toward African trypanosomes. *Proc. Natl. Acad. Sci. U. S. A.* 1994; 91:9735–9739. [PubMed: 7937882]

59. Price HP, et al. Myristoyl-CoA:protein N-myristoyltransferase, an essential enzyme and potential drug target in kinetoplastid parasites. *J. Biol. Chem.* 2003; 278:7206–7214. [PubMed: 12488459]
60. Brannigan JA, et al. N-myristoyltransferase from *Leishmania donovani*: structural and functional characterisation of a potential drug target for visceral leishmaniasis. *J. Mol. Biol.* 2010; 396:985–999. [PubMed: 20036251]
61. Price HP, et al. Myristoyl-CoA:protein N-myristoyltransferase depletion in trypanosomes causes avirulence and endocytic defects. *Mol. Biochem. Parasitol.* 2010; 169:55–58. [PubMed: 19782106]
62. Doering TL, et al. An analog of myristic acid with selective toxicity for African trypanosomes. *Science.* 1991; 252:1851–1854. [PubMed: 1829548]
63. Frearson JA, et al. N-myristoyltransferase inhibitors as new leads to treat sleeping sickness. *Nature.* 2010; 464:728–732. [PubMed: 20360736]
64. Price HP, et al. Functional analysis of TbARL1, an N-myristoylated Golgi protein essential for viability in bloodstream trypanosomes. *J. Cell Sci.* 2005; 118:831–841. [PubMed: 15687105]
65. Tull D, et al. Membrane protein SMP-1 is required for normal flagellum function in *Leishmania*. *J. Cell Sci.* 2010; 123:544–554. [PubMed: 20086045]
66. Emmer BT, et al. Calflagin inhibition prolongs host survival and suppresses parasitemia in *Trypanosoma brucei* infection. *Eukaryot. Cell.* 2010; 9:934–942. [PubMed: 20418379]
67. Sahin A, et al. The leishmania ARL-1 and golgi traffic. *PLoS One.* 2008; 3:e1620. [PubMed: 18286177]
68. Tull D, et al. SMP-1, a member of a new family of small myristoylated proteins in kinetoplastid parasites, is targeted to the flagellum membrane in *Leishmania*. *Mol. Biol. Cell.* 2004; 15:4775–4786. [PubMed: 15342784]
69. MacLean LM, et al. Trafficking and release of *Leishmania* metacyclic HASPB on macrophage invasion. *Cell. Microbiol.* 2012; 14:740–761. [PubMed: 22256896]
70. Stegmayer C, et al. Direct transport across the plasma membrane of mammalian cells of *Leishmania* HASPB as revealed by a CHO export mutant. *J. Cell Sci.* 2005; 118:517–527. [PubMed: 15657075]
71. Emmer BT, et al. Identification of a palmitoyl acyltransferase required for protein sorting to the flagellar membrane. *J. Cell Sci.* 2009; 122:867–874. [PubMed: 19240115]
72. Maric D, et al. Molecular determinants of ciliary membrane localization of *Trypanosoma cruzi* flagellar calcium-binding protein. *J. Biol. Chem.* 2011; 286:33109–33117. [PubMed: 21784841]
73. Godsel LM, Engman DM. Flagellar protein localization mediated by a calcium-myristoyl/palmitoyl switch mechanism. *EMBO J.* 1999; 18:2057–2065. [PubMed: 10205160]
74. Liu W, et al. Expression and cellular localisation of calpain-like proteins in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 2010; 169:20–26. [PubMed: 19766148]
75. Proto WR, et al. *Trypanosoma brucei* metacaspase 4 is a pseudopeptidase and a virulence factor. *J. Biol. Chem.* 2011; 286:39914–39925. [PubMed: 21949125]
76. de Paulo Martins V, et al. Acylation-dependent export of *Trypanosoma cruzi* phosphoinositide-specific phospholipase C to the outer surface of amastigotes. *J. Biol. Chem.* 2010; 285:30906–30917. [PubMed: 20647312]
77. Tull D, et al. Acylation-dependent and-independent membrane targeting and distinct functions of small myristoylated proteins (SMPs) in *Leishmania major*. *Int. J. Parasitol.* 2012; 42:239–247. [PubMed: 22281304]
78. Sunter J, et al. Determinants of GPI-PLC localisation to the flagellum and access to GPI-anchored substrates in trypanosomes. *PLoS Pathog.* 2013; 9:e1003566. [PubMed: 23990786]
79. Tyler KM, et al. Flagellar membrane localization via association with lipid rafts. *J. Cell Sci.* 2009; 122:859–866. [PubMed: 19240119]
80. Okura M, et al. A lipid-modified phosphoinositide-specific phospholipase C (TcPI-PLC) is involved in differentiation of trypomastigotes to amastigotes of *Trypanosoma cruzi*. *J. Biol. Chem.* 2005; 280:16235–16243. [PubMed: 15710612]

81. Olego-Fernandez S, et al. Cell morphogenesis of *Trypanosoma brucei* requires the paralogous, differentially expressed calpain-related proteins CAP5.5 and CAP5.5V. *Protist.* 2009; 160:576–590. [PubMed: 19656721]
82. Paturiaux-Hanocq F. A Role for the dynamic acylation of a cluster of cysteine residues in regulating the activity of the glycosylphosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. *J. Biol. Chem.* 2000; 275:12147–12155. [PubMed: 10766850]
83. Panethymitaki C, et al. Characterization and selective inhibition of myristoyl-CoA: protein N-myristoyltransferase from *Trypanosoma brucei* and *Leishmania major*. *Biochem. J.* 2006; 396:277–285. [PubMed: 16480339]
84. Sheng C, et al. Homology modeling and molecular dynamics simulation of N-myristoyltransferase from protozoan parasites: active site characterization and insights into rational inhibitor design. *J. Comput. Aided Mol. Des.* 2009; 23:375–389. [PubMed: 19370313]
85. Bell AS, et al. Selective inhibitors of protozoan protein N-myristoyltransferases as starting points for tropical disease medicinal chemistry programs. *PLoS Negl. Trop. Dis.* 2012; 6:e1625. [PubMed: 22545171]
86. Brand S, et al. Discovery of a novel class of orally active trypanocidal N-myristoyltransferase inhibitors. *J. Med. Chem.* 2011; 55:140–152. [PubMed: 22148754]
87. Adam JR, et al. Biochemical and genetic characterisation of *Trypanosoma cruzi* N-myristoyltransferase. *Biochem. J.* 2014
88. Beck JR, et al. A *Toxoplasma* palmitoyl acyl transferase and the palmitoylated Armadillo Repeat protein TgARO govern apical rhoptry tethering and reveal a critical role for the rhoptries in host cell invasion but not egress. *PLoS Pathog.* 2013; 9:e1003162. [PubMed: 23408890]
89. Georgopapadakou NH. Antifungals targeted to protein modification: focus on protein N-myristoyltransferase. *Expert Opin. Investig. Drugs.* 2002; 11:1117–1125.
90. Prasad KK, et al. N-myristoyltransferase: a novel target. *Mini Rev. Med. Chem.* 2008; 8:142–149. [PubMed: 18289097]
91. Sikorski JA, et al. Selective peptidic and peptidomimetic inhibitors of *Candida albicans* myristoylCoA: protein N-myristoyltransferase: a new approach to antifungal therapy. *Biopolymers.* 1997; 43:43–71. [PubMed: 9174411]
92. Goncalves V, et al. A fluorescence-based assay for N-myristoyltransferase activity. *Anal. Biochem.* 2012; 421:342–344. [PubMed: 22051857]
93. Jennings BC, Linder ME. DHHC protein S-acyltransferases use similar ping-pong kinetic mechanisms but display different acyl-CoA specificities. *J. Biol. Chem.* 2012; 287:7236–7245. [PubMed: 22247542]
94. Gelb MH, et al. Protein farnesyl and N-myristoyl transferases: piggy-back medicinal chemistry targets for the development of antitrypanosomatid and antimalarial therapeutics. *Mol. Biochem. Parasitol.* 2003; 126:155–163. [PubMed: 12615314]
95. Buckner FS, Eastman RT, Yokoyama K, Gelb MH, Van Voorhis WC. Protein farnesyl transferase inhibitors for the treatment of malaria and African trypanosomiasis. *Curr. Opin. Invest. Drugs.* 2005; 6:791–797.
96. Ferguson MA, et al. Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 1985; 260:14547–14555. [PubMed: 4055788]
97. Werbovets KA, Englund PT. Lipid metabolism in *Trypanosoma brucei*: utilization of myristate and myristoyllysophosphatidylcholine for myristoylation of glycosyl phosphatidylinositols. *The Biochemical journal.* 1996; 318:575–581. Pt 2. [PubMed: 8809049]
98. Schneider P, et al. Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease. *J. Biol. Chem.* 1990; 265:16955–16964. [PubMed: 2145267]
99. Ralton JE, McConville MJ. Delineation of three pathways of glycosylphosphatidylinositol biosynthesis in *Leishmania mexicana* precursors from different pathways are assembled on distinct pools of phosphatidylinositol and undergo fatty acid remodeling. *J. Biol. Chem.* 1998; 273:4245–4257. [PubMed: 9461623]

100. Masterson WJ, et al. Fatty acid remodeling: a novel reaction sequence in the biosynthesis of trypanosome glycosyl phosphatidylinositol membrane anchors. *Cell*. 1990; 62:73–80. [PubMed: 1694728]
101. Buxbaum LU, Milne KG, Werbovetz KA, Englund PT. Myristate exchange on the Trypanosoma brucei variant surface glycoprotein. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:1178–1183. [PubMed: 8577736]
102. McDowell M, et al. Glycosylphosphatidylinositol-dependent secretory transport in Trypanosoma brucei. *Biochem. J.* 1998; 335:681–689. [PubMed: 9794811]
103. Böhme U, Cross GA. Mutational analysis of the variant surface glycoprotein GPI-anchor signal sequence in Trypanosoma brucei. *J. Cell Sci.* 2002; 115:805–816. [PubMed: 11865036]
104. Pal A, et al. Rab5 and Rab11 mediate transferrin and anti-variant surface glycoprotein antibody recycling in Trypanosoma brucei. *Biochem. J.* 2003; 374:443–451. [PubMed: 12744719]
105. Sevova ES, Bangs JD. Streamlined architecture and glycosylphosphatidylinositol-dependent trafficking in the early secretory pathway of African trypanosomes. *Mol. Biol. Cell.* 2009; 20:4739–4750. [PubMed: 19759175]
106. Triggs VP, Bangs JD. Glycosylphosphatidylinositol-dependent protein trafficking in bloodstream stage Trypanosoma brucei. *Eukaryot. Cell.* 2003; 2:76–83. [PubMed: 12582124]
107. Schwartz KJ, et al. GPI valence and the fate of secretory membrane proteins in African trypanosomes. *J. Cell Sci.* 2005; 118:5499–5511. [PubMed: 16291721]
108. Jaquenoud M, et al. The Gup1 homologue of Trypanosoma brucei is a GPI glycosylphosphatidylinositol remodelase. *Mol. Microbiol.* 2008; 67:202–212. [PubMed: 18036137]
109. Engstler M, et al. Overath P. Hydrodynamic flow-mediated protein sorting on the cell 530 surface of trypanosomes. *Cell*. 2007; 131:505–515. N. 529 Heddergott. [PubMed: 17981118]
110. Field MC, et al. Macromolecular trafficking and immune evasion in African trypanosomes. *Int. Rev. Cell Mol. Biol.* 2009; 278:1–67. [PubMed: 19815176]
111. Rifkin MR, Landsberger FR. Trypanosome variant surface glycoprotein transfer to target membranes: a model for the pathogenesis of trypanosomiasis. *Proc. Natl. Acad. Sci.* 1990; 87:801–805. [PubMed: 2300563]
112. Gruszynski AE, et al. Surface coat remodeling during differentiation of Trypanosoma brucei. *J. Biol. Chem.* 2003; 278:24665–24672. [PubMed: 12716904]
113. Magez S, et al. The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors. *J. Immunol.* 1998; 160:1949–1956. [PubMed: 9469458]
114. Paulnock DM, et al. Modulation of innate immunity by African trypanosomes. *Parasitology*. 2010; 137:2051–2063. [PubMed: 21087532]
115. Heng J, et al. Membrane targeting of the small myristoylated protein 2 (SMP-2) in Leishmania major. *Mol. Biochem. Parasitol.* 2013; 190:1–5. [PubMed: 23727225]

Highlights

- Fatty acylation is essential in trypanosomatids.
- Inhibition of fatty acylation is a promising avenue for development of trypanocidal drugs.
- Acyl modifications are critical determinants of protein trafficking and localization.
- Some myristoylation inhibitors are highly specific for the trypanosomatid enzymes.

Figure 1.

Myristoylation and palmitoylation. Myristoylation: N-myristoyl transferase (NMT) binds to myristoyl-CoA and the recognition sequence (Figure 2) of its substrate protein. Nucleophilic substitution of the N-terminal glycine amine with myristate leads to the production of myristoylated protein and CoA-SH. Palmitoylation: palmitoylation is catalyzed by the enzyme palmitoyl acyltransferase (PAT), which is reversed by the action of a thioesterase. PAT binds to palmitoyl-CoA and the recognition sequence (Figure 2) of its substrate protein via its DHHC catalytic site. PAT first becomes autopalmitoylated and then transfers the palmitate to the substrate protein to generate palmitoylated protein and CoA-SH.

Myristoylation Site

1	2	3	4	5	6	7	8	9
Met	Gly	Small Un-charged	X	X	Ser Preferred	No Pro or Asp	Basic > Neutral > Acidic	Basic > Neutral > Acidic

Palmitoylation Site

Type I – X-CC-X Pattern	X	Cys	Cys	X		
Type II – CXXC Pattern	X	Cys	X	X	Cys	X
Type III – Random Pattern	X	Cys	X			

Figure 2.

Recognition sequences for myristoylation and palmitoylation. Protein myristoylation occurs on the N-terminal glycine residue at position 2 (red), with additional aspects of the sequence motif indicated in positions 3-9, where X represents any amino acid. Protein palmitoylation occurs at three types of sites, which may be located throughout the polypeptide sequence: Type I (XCCX), Type II (CXXC), or Type III (Random) pattern, where X represents any amino acid. The X amino acid residues in palmitoylation sites are preferentially neutral > basic > acidic.

Figure 3.

Hypothetical trafficking pattern of dually acylated proteins. Hypothetical pathway for an exemplar dually acylated myristoylated/palmitoylated trypanosomatid protein, calflagin/FCaBP, based in part on data in [71] and [72]. (1) Newly-synthesized protein (blue) is myristoylated by NMT and (2) associates with vesicles in the Golgi, perhaps having a distinct lipid composition specifying flagellar pocket (FP) targeting. (3) The vesicles traffic to and fuse with the flagellar pocket. (4) If the protein is not palmitoylated by PAT (orange, shown here in the FP), it trafficks to the pellicular membrane. (5) If the protein is palmitoylated, it has two possible fates, depending on whether it also possesses a polybasic region (KKKK). (6) If KKKK is present, the protein enters the flagellar compartment and associates with the flagellar membrane. (7) If KKKK is absent, it continues onto the pellicular membrane. This is only one model for trafficking of one protein. Additional known and possible factors specifying trafficking of acylated proteins include protein-protein interactions, the specific lipid chemistry of distinct membrane domains, such as the flagellar membrane [79], and the location of the specific PAT.

Table 1

Predicted PATs in trypanosomatids

PAT Family	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
<i>T. brucei</i>	.	.	.	.	.	.	.	.	.	.	.	.					
<i>T. cruzi</i>	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
<i>L. major</i>	.	.	.	.	.	.	.	.	.	.	.	...	.	.	.	.	.

• indicate homologs of each PAT type; numbering originally established in [71]

Table 2

Summary of trafficking pathways

Protein	Species	Acylation state	Location after myristoylation	Location after palmitoylation	Function	Ref.
ARL-1	<i>T. brucei</i> , <i>L. major</i>	myristoyl	Golgi	N/A	endocytosis, protein trafficking, Golgi structure	[64, 67]
SMP-1	<i>Leishmania</i>	myristoyl, palmitoyl	golgi	flagellum	flagellar structure, stability, motility	[65, 68, 77]
SMP-2	<i>Leishmania</i>	myristoyl	flagellar pocket	N/A	unknown	[65, 77, 115]
SMP-4	<i>Leishmania</i>	myristoyl	pellicular membrane	pellicular membrane (palm site introduced)	unknown	[65, 77]
HASPB	<i>Leishmania</i>	myristoyl, palmitoyl	golgi	flagellum	metacyclogenesis; promastigote localization in fly	[69]
Calflagin	<i>T. brucei</i>	myristoyl, palmitoyl	pellicular membrane	flagellum	calcium binding	[71]
CAP 5.5	<i>T. brucei</i>	myristoyl, palmitoyl	unknown	flagellum	calpain-like protein; cell morphogenesis	[44]
CALP 1.3	<i>T. brucei</i>	myristoyl, palmitoyl	unknown	flagellum	calpain-like protein	
MCA4	<i>T. brucei</i>	myristoyl, palmitoyl	unknown	flagellum	Cell cycle progression, virulence during infection	[75]
FCaBP	<i>T. cruzi</i>	myristoyl, palmitoyl	pellicular membrane	flagellum	calcium binding	[72]
PIPLC	<i>T. cruzi</i>	myristoyl, palmitoyl	golgi	pellicular membrane	differentiation	[76, 80]
PPEF	<i>Leishmania</i>	myristoyl, palmitoyl	golgi	flagellum	unknown	[41]
GPI- PLC	<i>T. brucei</i>	dual; s- myristoylation	unknown	plasma membrane, flagellum	release of VSG from differentiating cells; rapid coat switching	[78, 82]