# Identification of a palmitoyl acyltransferase required for protein sorting to the flagellar membrane

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## Summary

**Protein palmitoylation has diverse effects in regulating protein membrane affinity, localization, binding partner interactions, turnover and function. Here, we show that palmitoylation also contributes to the sorting of proteins to the eukaryotic flagellum. African trypanosomes are protozoan pathogens that express a family of unique $Ca^{2+}$-binding proteins, the calflagins, which undergo N-terminal myristoylation and palmitoylation. The localization of calflagins depends on their acylation status. Myristoylation alone is sufficient for membrane association, but, in the absence of palmitoylation, the calflagins localize to the pellicular (cell body) membrane. Palmitoylation, which is mediated by a specific palmitoyl acyltransferase, is then required for subsequent trafficking of calflagin to the flagellar membrane. Coincident with the redistribution of calflagin from the pellicular to the flagellar membrane is their association with lipid rafts, which are highly enriched in the flagellar membrane. Screening of candidate palmitoyl acyltranferases identified a single enzyme, TbPAT7, that is necessary for calflagin palmitoylation and flagellar membrane targeting. Our results implicate protein palmitoylation in flagellar trafficking, and demonstrate the conservation and specificity of palmitoyl acyltransferase activity by DHHC-CRD proteins across kingdoms.**

Key words: Flagellum, Palmitoylation, Membrane microdomains, Acylation, Trypanosoma

## Introduction

Protein palmitoylation is the thioester linkage of long chain fatty acids, most commonly palmitic acid, to cysteine residues of protein substrates. Palmitoylation was first described nearly four decades ago (Braun and Radin, 1969), but only recently have the discoveries of the first protein palmitoyl acyltransferases (PATs) in yeast (Lobo et al., 2002; Roth et al., 2002) led to rapid progress in understanding the nature and functions of this important protein modification (Linder and Deschenes, 2007). PAT activity is found in proteins with a signature aspartate-histidine-histidine-cysteine cysteine-rich domain, the DHHC-CRD, which mediates enzymatic activity through the formation of a palmitoyl-enzyme intermediate (Mitchell et al., 2006). Palmitoylation is remarkable among protein lipid modifications in that the modification often takes place on proteins already localized to a membrane. In addition to its ability to strengthen this membrane association, palmitoylation can regulate broad aspects of protein localization and function. In particular, protein sorting between different membrane domains can be modulated by palmitoylation (Greaves and Chamberlain, 2007).

The kinetoplastid parasite *Trypanosoma brucei* is the etiologic agent of African sleeping sickness, a blood-borne infection that is fatal if untreated. Aside from the tremendous global health burden it causes, *T. brucei* is an excellent model organism for studying the eukaryotic flagellum/cilium (Kohl and Bastin, 2005). *T. brucei* cells are amenable to genetic manipulation and possess a single polarized flagellum that drives cellular motility and compartmentalizes signaling proteins. We and others have shown the trypanosome pellicular (cell body) membrane and flagellar membrane to have distinct lipid compositions (Kaneshiro, 1990; Tetley, 1986). Specifically, we have found the flagellar membrane to be enriched in lipid rafts: regions of increased membrane order that may serve as signaling or recruitment platforms for associated molecules (Tyler et al., 2009). Association with lipid rafts in the flagellar membrane might therefore provide a general mechanism by which specific proteins traffic to the flagellar/ciliary membrane.

A family of proteins that might be expected to use such a mechanism is the calflagin family of $Ca^{2+}$-binding proteins. These proteins localize to the flagellar membrane but not to the pellicular membrane. They were discovered as the predominant molecules in a *T. brucei* lysate to bind a hydrophobic resin in a $Ca^{2+}$-dependent manner (Wu et al., 1992). Three proteins, Tb44, Tb24 and Tb17, comprise the calflagin family (Wu et al., 1994), each containing multiple EF-hand domains that bind $Ca^{2+}$ to induce subsequent conformational changes. The calflagins are important for parasite virulence in an animal model of infection, but the molecular basis for this observation is not yet established (our unpublished results).

In this study, we sought to determine the impact of palmitoylation on calflagin biology, and to identify the molecular components involved in this modification. Our results demonstrate that palmitoylation of calflagins by a single DHHC-CRD enzyme, TbPAT7, promotes association with lipid raft microdomains and redirects the calflagins from the pellicular to the flagellar membrane. These findings add flagellar trafficking to the list of functions ascribed to protein palmitoylation and the calflagins to the list of proteins for which a dedicated palmitoyl acyltransferase has been identified.

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## Results

### Detection of palmitoylation by acyl-biotin exchange chemistry

To test for protein palmitoylation, *T. brucei* procyclic stage extracts were subjected to an acyl-biotin exchange reaction. In this assay, cell lysates are treated with hydroxylamine to cleave protein-palmitoyl linkages and generate reactive thiol groups, which are then labeled with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). Biotinylated proteins are then purified by streptavidin-agarose chromatography. Non-specific labeling of free thiols is blocked by pre-incubation of the extract with N-ethylmaleimide before hydroxylamine treatment, and residual background labeling and purification is monitored by treating samples in parallel with Tris-HCl in place of hydroxylamine. This technique was recently used in yeast to establish the first palmitoylproteome (Roth et al., 2006).

Application of this approach to *T. brucei* demonstrated hydroxylamine-dependent purification of the positive control CAP5.5 protein, which was previously shown to be palmitoylated by $^{3}$H-palmitate radiolabeling (Hertz-Fowler et al., 2001), but none of the control proteins from the cytoskeleton [β-tubulin, WCB (Woods et al., 1992)], endoplasmic reticulum [BiP (Bangs et al., 1993)], plasma membrane (procyclin) or cytoplasm (Hsp70) (Fig. 1). Like CAP5.5, all three calflagin proteins were biotinylated and purified by streptavidin under experimental but not control conditions. The calflagin-specific antiserum also detected a cross-reactive protein of 38 kDa in acyl-biotin exchange samples, but this protein was not purified by streptavidin under any conditions. The hydroxylamine-dependent purification of calflagins Tb44, Tb17 and Tb24 by acyl-biotin exchange and streptavidin chromotagraphy indicates that these proteins are in fact palmitoylated in procyclic stage *T. brucei* cells.

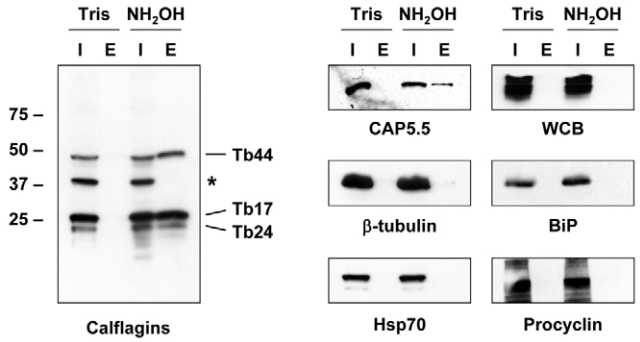


**Fig. 1.** *T. brucei* calflagins are palmitoylated in vivo. Palmitoylated proteins were specifically labeled in *T. brucei* lysates by cleaving fatty acid-thioester bonds with hydroxylamine and labeling the liberated thiols with biotin-HPDP, as described in the Materials and Methods. Acyl-biotin exchange samples from control (Tris) and hydroxylamine ($NH_2OH$) treatment conditions were then subjected to streptavidin affinity chromatography to purify palmitoylated proteins. Samples from each input (I) and eluate (E) fraction were analyzed by immunoblotting. A control palmitoylated protein (CAP5.5) was purified in a hydroxylamine-dependent manner, whereas negative controls from the cytoskeleton (β-tubulin), cytoplasm (Hsp70), endoplasmic reticulum (BiP) and cell membrane (procyclin) were not. The calflagin antiserum identified three bands corresponding to Tb44, Tb17 and Tb24 (indicated) as well as a cross-reactive protein (asterisk) of 38 kDa in the streptavidin input. Only the calflagin proteins were purified by streptavidin under hydroxylamine treatment.

### Distinct roles of myristoylation and palmitoylation in calflagin trafficking

To identify the specific residues modified by acylation and to investigate the consequences of disrupted acylation on calflagin trafficking, we performed site-directed mutagenesis of individual calflagin amino acids. The N-terminus has a high degree of sequence identity among the calflagins that is shared to a lesser extent with the *T. cruzi* flagellar $Ca^{2+}$-binding protein (FcaBP) (Fig. 2A), which is also dually acylated and exhibits acylation-dependent flagellar membrane targeting (Godsel and Engman, 1999). We engineered constructs for expression of wild-type and mutant calflagin Tb44 in *T. brucei*. A C-terminal myc tag was included to distinguish the transprotein from endogenous calflagins. The myristoylation state of the tagged protein was monitored by $^{3}$H-myristate labeling and the palmitoylation state by acyl-biotin exchange. Substitution of the glycine residue in the 2nd position with alanine prevented both acyl modifications, while substitution of the cysteine residue in the 3rd position with alanine abrogated palmitoylation but left myristoylation intact, as expected (Fig. 2B).

Immunofluorescence microscopy revealed the wild-type protein to be flagellar (Fig. 3), identical to what is seen with calflagin antiserum staining of untransfected cells. The G2A mutant, which is neither myristoylated nor palmitoylated, was found diffusely throughout the cytoplasm. The C3A mutant, which is myristoylated but not palmitoylated, localized to the pellicular membrane but not the flagellar membrane. Myristoylation, therefore, which occurs co-translationally in the endoplasmic reticulum, promotes plasma membrane association for the calflagins. Palmitoylation, which occurs post-translationally at an unknown site and requires prior myristoylation, then provides a secondary signal necessary for calflagin sorting to the flagellar membrane.

Because of the different membrane localizations of dually-acylated and myristoylated-only calflagin, and because of the enrichment of lipid rafts in the flagellar membrane, we next tested for the dependence of calflagin lipid raft association on acylation. Two independent assays were used to test for raft association. First, parasites were treated with 1% Triton X-100 and fractionated into supernatant and pellet fractions at 4°C and 37°C. Lipid raft-associated proteins exhibit a temperature dependence in this assay, fractionating in the pellet and supernatant at 4°C but in the supernatant only at 37°C. Second, parasite lysates were analyzed by discontinuous sucrose density gradient centrifugation. Lipid raft-associated proteins are more buoyant, and are found in the low density interface fraction (fraction 2 in this assay). By both analyses, wild-type calflagin, but neither G2A nor C3A mutant calflagins, exhibited properties that indicated lipid raft association (Fig. 4). Myristoylation, therefore, although sufficient for plasma membrane localization, is insufficient for partitioning into lipid rafts. Only the mature protein with both acyl modifications is found in lipid rafts.

### Screening of *T. brucei* DHHC-CRD containing genes uncovers a single enzyme necessary for calflagin palmitoylation in vivo

Given the importance of palmitoylation in conferring flagellar localization to the calflagins, we set out to identify the enzyme catalyzing this reaction. The availability of the *T. brucei* genome sequence (www.genedb.org) allowed us to identify all of the genes containing the DHHC-CRD characteristic of palmitoyl acyltransferases in yeast and mammalian cells. Twelve such genes are found in the *T. brucei* genome (Table 1). These genes are predicted to encode proteins ranging in size between 30 and 90 kDa, all with multiple transmembrane domains and conserved in other kinetoplastid parasites.

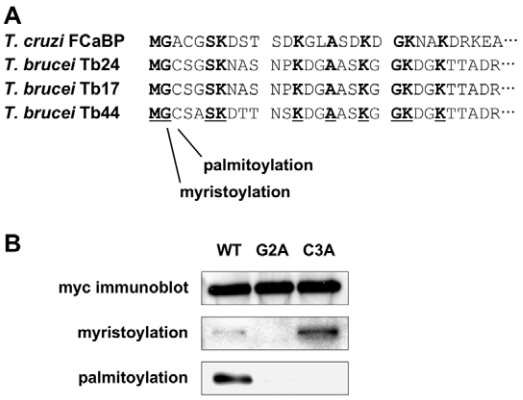


**Fig. 2.** N-terminal mutagenesis abolishes acylation. (A) The N-terminal sequence is highly conserved among the calflagins and is very similar to that of the dually acylated flagellar $Ca^{2+}$-binding protein (FCaBP) of *T. cruzi*. Each protein contains an N-terminal glycine followed closely by a cysteine residue, the sites of myristoylation and palmitoylation, respectively. Residues conserved in each protein are shown in bold and underlined. (B) The wild-type Tb44 open reading frame was cloned into the pLEW79-Myc vector for parasite expression with a C-terminal epitope tag. Expression was analyzed by anti-myc immunoblotting of $5\times10^6$ cell equivalents of lysate. The wild-type protein underwent myristoylation and palmitoylation, as detected by metabolic labeling and acyl-biotin exchange chemistry, respectively. Mutations were introduced into the predicted sites of acylation. The G2A mutant lacked both myristoylation and palmitoylation, whereas the C3A mutant was myristoylated but not palmitoylated.

Inhibition of candidate PATs was performed by generating individual parasite strains inducible for RNA interference of each protein. Target sequences were selected using RNAit software, which scans the open reading frame to identify regions with minimal similarity to off-target genes (Redmond et al., 2003). The target regions were amplified from *T. brucei* genomic DNA and subcloned into the pZJM vector, which provides flanking opposing T7 promoters and sequences for integration into a rDNA spacer region (Wang et al., 2000). Constructs for each candidate PAT were then transfected into a *T. brucei* procyclic line expressing both bacteriophage T7 RNA polymerase and tet repressor, enabling drug-inducible production of dsRNA (Wirtz et al., 1999). Inducible RNAi cell lines were generated for each of the twelve candidate PATs, and semi-quantitative RT-PCR verified inhibition of each candidate PAT (data not shown). None of the targeted genes proved to be essential, as parasites continued to divide in culture in all cases. The gross morphology and flagellar motility furthermore appeared unaffected in each mutant. These findings suggest either that no single DHHC-CRD protein is required for parasite viability, proliferation or motility in procyclic stage trypanosomes, or that residual amounts of protein remaining after RNA interference are sufficient to prevent a discernible phenotype.

We screened these twelve mutants for calflagin localization by immunofluorescence microscopy. Eleven of the 12 mutants showed clear flagellar localization for calflagin, indistinguishable from wild-type cells. However, for a single RNAi target, TbPAT7, drug induction resulted in mislocalization of calflagin to the pellicular membrane (Fig. 5), identical to our calflagin C3A mutant (Fig. 3). The effect was highly specific, as ~95% of TbPAT7 RNAi cells mislocalized calflagin after 48 hours, whereas none of the other 11 mutants lines yielded >5% of cells with mislocalized calflagin (Fig. 6A). Analysis of the kinetics of calflagin mislocalization showed a $t_{1/2}$ of ~24 hours (Fig. 6B). Mislocalization of calflagin by TbPAT7 depletion was reversible, as removal of tetracycline at 96 hours allowed calflagin to localize properly to the flagellar membrane.

As our screen relied on calflagin mislocalization as a surrogate for calflagin palmitoylation, it remained possible that, rather than affecting calflagin palmitoylation, TbPAT7 RNAi interfered with another aspect of protein trafficking. To test for a direct effect of

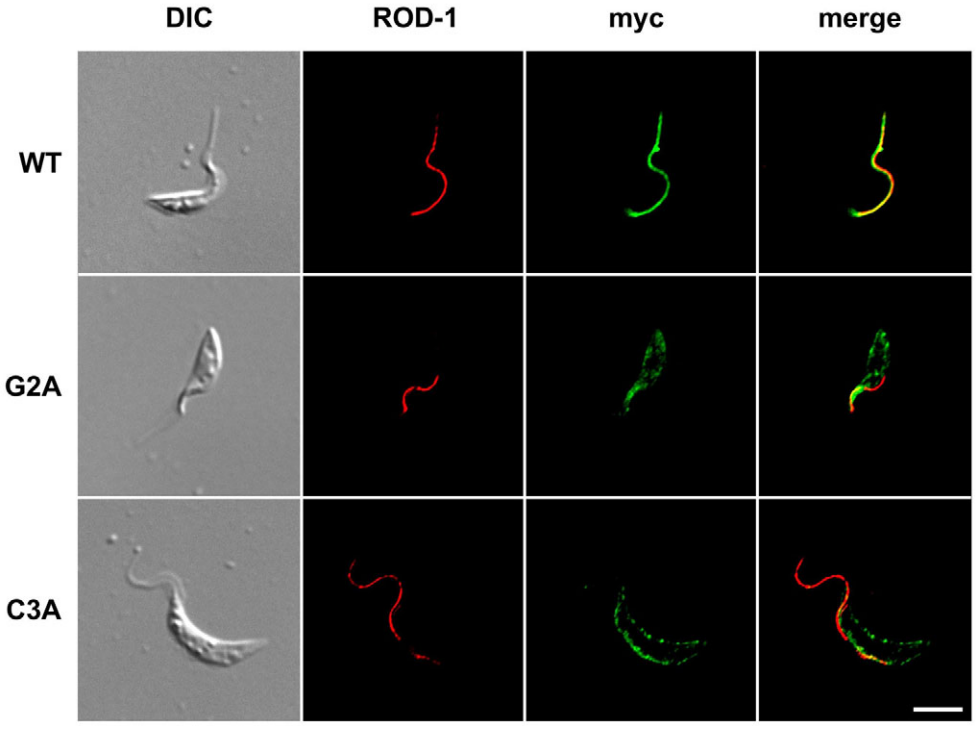


**Fig. 3.** Localization of acylation mutants. Parasites expressing myc-tagged wild-type (WT), G2A (nonacylated) or C3A (myristoylated) calflagin Tb44 were examined by differential interference contrast (DIC) microscopy and calflagin (myc)-specific (green) or paraflagellar rod (ROD-1)-specific (red) immunofluorescence microscopy. Bar, 5 µm. The wild-type protein is flagellar, the G2A mutant protein is found throughout the cell and the C3A mutant localizes to the pellicular membrane.

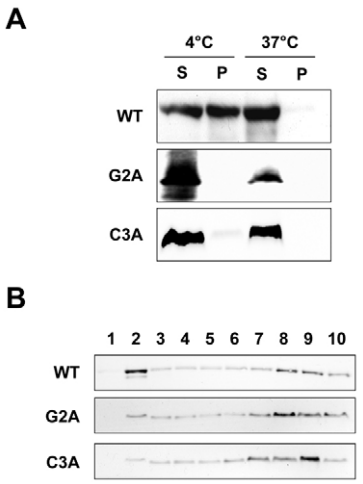


**Fig. 4.** Mutations that abolish palmitoylation also disrupt association with lipid rafts. (A) Parasites were fractionated into supernatant (S) and pellet (P) fractions in PBS + 1% Triton X-100 at either 4°C or 37°C as described in the Materials and Methods. The wild-type dually acylated Tb44 protein, but neither acylation mutant, exhibited temperature-dependent detergent resistance. (B) Parasite extracts were loaded at the bottom (Fraction 10) of a discontinuous Optiprep density gradient and subjected to ultracentrifugation. Fractions were collected and analyzed by anti-myc immunoblotting. Fraction 2 contains the lipid raft interface. The wild-type dually acylated Tb44 protein, but neither acylation mutant, floated to the lipid raft interface.

TbPAT7 depletion on calflagin palmitoylation, we performed acyl-biotin exchange reactions on wild-type and TbPAT7 mutant cells (Fig. 7). Indeed, calflagin palmitoylation was disrupted in a time frame consistent with calflagin mislocalization. Calflagins were undetectable in streptavidin eluates prepared from TbPAT7-depleted lysates harvested 48 hours post-induction. TbPAT7 is thus necessary for the in vivo palmitoylation of calflagins in procyclic stage *T. brucei*.

## Discussion

There are several notable differences between palmitoylation and other covalent lipid modifications of proteins. For example, whereas myristoylation is irreversible, co-translational and catalyzed by a single endoplasmic-reticulum-localized enzyme (N-myristoyltransferase) (Farazi et al., 2001), palmitoylation is reversible, post-translational and catalyzed by multiple enzymes at sites throughout the cell. Many palmitoylated proteins already localize to a membrane prior to their palmitoylation, either through transmembrane domains or other lipid modifications. Palmitoylation can then strengthen that association, as well as confer other functions. Perhaps the best studied palmitoylated proteins are the mammalian Ras oncoproteins. Farnesylation of H-Ras and N-Ras in the endoplasmic reticulum confers membrane association, whereas secondary palmitoylation/depalmitoylation regulates the cycling of these proteins between the plasma membrane and Golgi apparatus (Rocks et al., 2005).

Our results implicate palmitoylation in another cellular function: the trafficking of proteins to the eukaryotic flagellar membrane. The pellicular membrane localization of calflagins upon inhibition of palmitoylation, whether by point mutagenesis or by depletion of their cognate PAT, indicates that palmitoylation is required for calflagin sorting to the flagellar membrane. There are several reports of proteins in other organisms for which palmitoylation likewise regulates sorting to specific membrane domains. In neurons, the concentration of PSD-95 at postsynaptic clusters (Craven et al., 1999), synaptotagmin I at presynaptic sites (Kang et al., 2004) and GAP-43 at axonal membranes (El-Husseini Ael et al., 2001) all require palmitoylation.

**Table 1. Identification of candidates for calflagin palmitoylation**

| | GeneDb accession number | Predicted number of amino acids | DHHC-CRD motif |
|---|---|---|---|
| TbPAT1 | Tb09.160.5650 | 820 | 502-566 |
| TbPAT2 | Tb10.406.0080 | 452 | 116-180 |
| TbPAT3 | Tb10.70.7520 | 334 | 111-171 |
| TbPAT4 | Tb11.01.0810 | 469 | 272-336 |
| TbPAT5 | Tb11.01.4490 | 452 | 207-272 |
| TbPAT6 | Tb11.02.1710 | 471 | 249-313 |
| TbPAT7 | Tb11.02.4090 | 578 | 425-487 |
| TbPAT8 | Tb11.02.5140 | 267 | 91-155 |
| TbPAT9 | Tb11.02.5260 | 305 | 110-174 |
| TbPAT10 | Tb927.3.2400 | 307 | 112-176 |
| TbPAT11 | Tb927.7.3350 | 357 | 108-172 |
| TbPAT12 | Tb927.8.3400 | 297 | 123-187 |

Twelve genes are found in the *T. brucei* genome that contain the Pfam DHHC cysteine-rich domain (PF01529).

The extent to which palmitoylation-dependent protein sorting to the flagellum is shared by other flagellar membrane proteins in *T. brucei*, as well as in other flagellated and ciliated cells, remains to be seen. In the related parasite *Leishmania major*, SMP-1 also localizes to the flagellar membrane in a manner that requires N-terminal myristoylation and palmitoylation (Tull et al., 2004). Another *L. major* protein, LmPPEF, also contains an N-terminal peptide whose dual acylation is sufficient for flagellar membrane localization of a fusion protein (Mills et al., 2007). However, the full-length protein localizes to intracellular membranes by virtue of additional protein-protein interactions. Similarly, the *T. brucei* protein CAP5.5 (Hertz-Fowler et al., 2001) and the *T. cruzi* protein TcPI-PLC (Furuya et al., 2000; Okura et al., 2005) are dually acylated, yet localize to the pellicular membrane. For CAP5.5, this is believed to be mediated by a biochemical association with subpellicular microtubules. Therefore, although dual acylation in itself appears to elicit flagellar membrane association in kinetoplastid parasites, this signal can be superseded by additional cues.

Recently, great strides have been made in understanding the molecular basis of flagellar protein trafficking. Unlike proteins specific for organelles such as the endoplasmic reticulum and lysosome, there does not appear to be a single signal shared by a majority of flagellar proteins. Rather, a process called intraflageller transport (IFT), discovered in *Chlamydomonas reinhardtii* (Kozminski et al., 1993) but seemingly functional in nearly all ciliated/flagellated eukaryotic cells, plays a central role in trafficking proteins to and from the flagellum. In IFT, proteinaceous 'barges' associate with microtubule motors to move along the axoneme and deliver structural subunits to the flagellar distal tip. Inhibition of *T. brucei* IFT genes causes a variety of abnormal phenotypes, including loss of motility and defective flagellar biogenesis. Electron microscopy has detected these barges in flagellar cross-section, and live cell fluorescence microscopy has detected movement of IFT particles along the *T. brucei* flagellum (Absalon et al., 2008). However, it remains unclear whether IFT is important for transport of membrane proteins to and from the flagellum. In *Chlamydomonas*

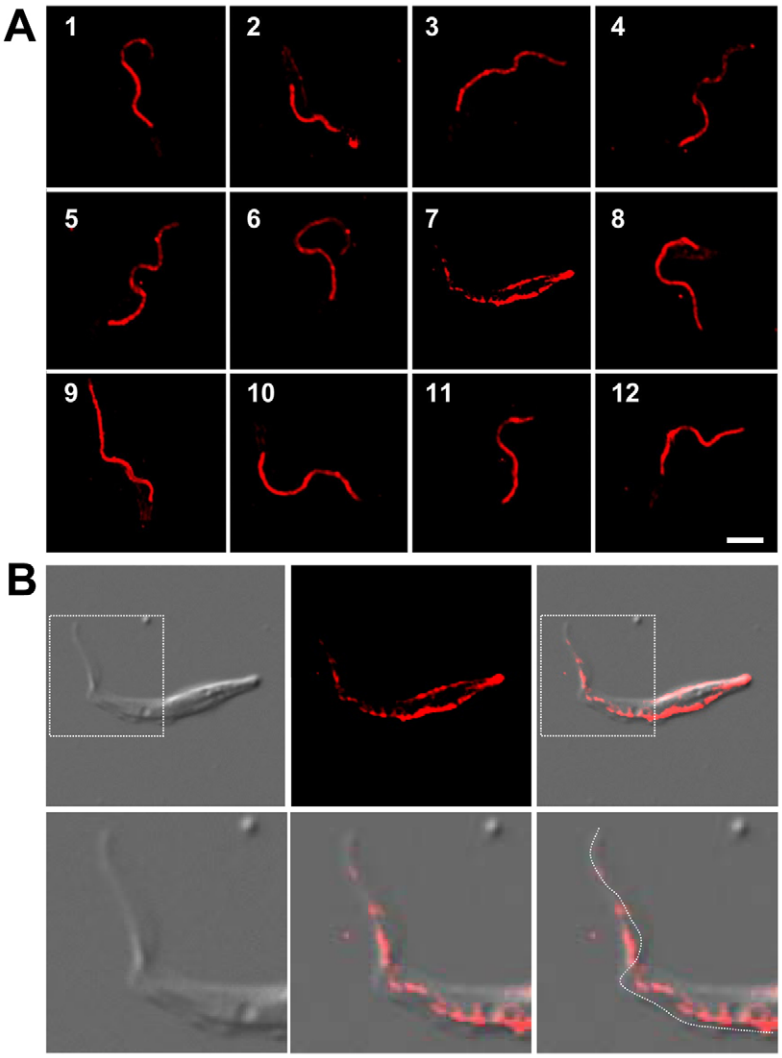


**Fig. 5.** Calflagins localize to the pellicular membrane upon inhibition of TbPAT7. RNAi against each candidate PAT was induced by the addition of tetracycline to cultured procyclic cells. Calflagin immunofluorescence microscopy was obtained 48 hours post-induction. (A) Representative cells of each mutant are shown, with the number in the top left corner of each box indicating the target TbPAT. Inhibition of TbPATs 1-6 and 8-12 had no effect on the flagellar localization of calflagin, whereas TbPAT7 RNAi resulted in calflagin localization to the pellicular membrane. Bar, 5 μm. (B) The TbPAT7 RNAi cell is shown at higher magnification together with DIC microscopy. Close inspection reveals that fluorescence is restricted to the pelliculum, with no fluorescence in the flagellum (outlined, lower right).

*reinhardtii*, at least two transmembrane proteins have been shown to be transported by IFT (Huang et al., 2007; Qin et al., 2005). However, multiple groups have observed the presence of a 'flagellar sleeve', an extension of nascent flagellar membrane arising from the basal body, even in the absence of axoneme assembly (Absalon et al., 2008; Davidge et al., 2006). This has led to the suggestion that membrane delivery to the flagellum may be independent of intraflagellar transport.

The simultaneous loss of calflagin lipid raft association and flagellar localization upon inhibition of palmitoylation is suggestive, but not proof, of lipid raft recruitment serving as a mechanism for palmitoylation-dependent calflagin trafficking. Palmitoylation of mammalian H-Ras on Cys181 similarly promotes an association with lipid raft microdomains (Roy et al., 2005). It is important to note that our results, which indicate a crucial role for palmitoylation in sorting to the flagellar membrane, do not exclude a role for palmitoylation in IFT. Palmitoylation, rather than simply redistributing protein along the membrane, might confer association of calflagins onto IFT barges. IFT barges appear to have a tight association with membrane components, as is evident in transmission electron micrographs (Kozminski et al., 1995). However, the detergent treatments involved in purification of IFT barges have precluded identification of their membrane components. Recent evidence from our laboratory suggests that membrane associated with IFT barges might be enriched in lipid raft components (Tyler et al., 2009). Detergent extraction of *T. brucei* cells revealed distinct patches of detergent-resistant membrane along the flagellum. Intriguingly, these patches were regularly spaced along the axoneme, but not found anywhere along the adjacent paraflagellar rod. The size and distribution of these patches were consistent with their being the membrane components of IFT barges. These findings suggest that palmitoylation of calflagins might confer flagellar membrane targeting by facilitating their loading onto IFT barges.

A number of other possible pathways, however, are consistent with the notion that palmitoylation promotes calflagin targeting to the flagellar membrane. Human and yeast PATs are distributed at multiple sites throughout the cell, including the plasma membrane, Golgi apparatus and endoplasmic reticulum (Ohno et al., 2006). Calflagin palmitoylation might occur directly at the pellicular membrane, upon which calflagin would actively sort out of this domain into the flagellum, where the distinct lipid environment might be more permissive to dually acylated proteins. Alternatively, myristoylated proteins might, at a low rate, diffuse from the pellicular membrane into the flagellum independently of this modification. Palmitoylation might then occur in the flagellar

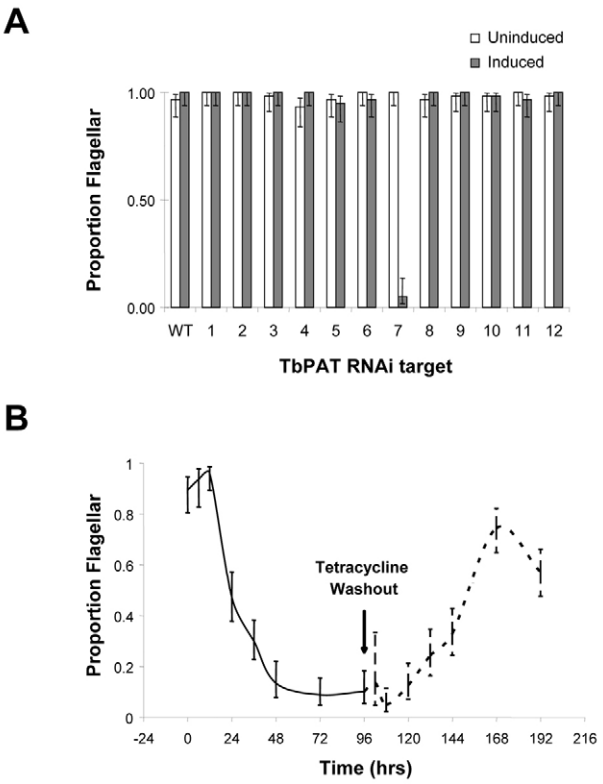


**Fig. 6.** Quantification and kinetics of calflagin mislocalization upon TbPAT7 RNAi. (A) The parental wild-type and all the PAT mutants were analyzed 48 hours post-induction of RNA interference by determining the proportion of cells showing flagellar localization of calflagins. Calflagin mislocalization was observed in ~95% of TbPAT7 RNAi cells but in no more than 5% of any other cell. (B) The proportion of TbPAT7 mutant cells showing flagellar localization of calflagins was analyzed during a time course of RNAi induction and release. At the indicated times post-induction, parasites were removed from the culture and fixed with paraformaldehyde. After 96 hours, tetracycline was washed out by three sequential rounds of centrifugation and resuspension in PBS (first two spins) or fresh medium. At the conclusion of the time course, all slides were analyzed by calflagin immunofluorescence microscopy. Calflagin mislocalization exhibited a $t_{1/2}$ of ~24 hours and was reversible upon tetracycline washout. Error bars indicate 95% confidence intervals. Between 40 and 100 individual cells were scored at each data point by calflagin immunofluorescence microscopy.

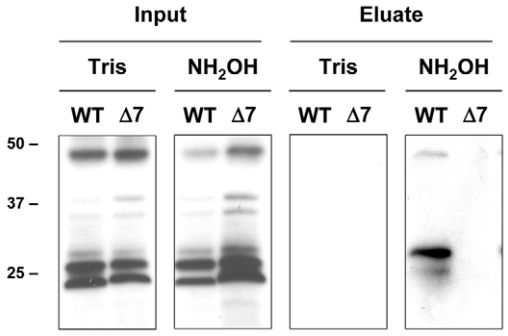


**Fig. 7.** Depletion of TbPAT7 inhibits calflagin palmitoylation. Lysates were harvested from wild-type (WT) and TbPAT7 mutant cells (Δ7) that were induced for RNAi for 48 hours and analyzed by acyl-biotin exchange and calflagin immunoblotting. The input material from Tris- and $NH_2OH$-treated samples is shown on the left and the streptavidin eluates from acyl-biotin exchange reactions are shown on the right. Only in wild-type cells are the calflagins palmitoylated, as determined by their ability to be biotinylated by the acyl-biotin exchange reaction in a $NH_2OH$-dependent manner, and subsequently purified by streptavidin and detected by immunoblotting with calflagin-specific antiserum.

membrane itself, where it would promote anchoring or retention of protein in this organelle. Finally, the pellicular membrane localization of non-palmitoylated calflagin might represent not an intermediate step of trafficking, but rather an alternative destination realized only upon inhibition of normal protein maturation. In this case, palmitoylation could occur at an earlier stage of the trafficking pathway, such as the Golgi apparatus or endoplasmic reticulum. Distinguishing among these possible pathways will be a focus of future investigation.

Finally, calflagin palmitoylation is remarkable for its degree of specificity. In yeast, palmitoylation of many proteins can be mediated by multiple PATs, such that prevention of individual protein palmitoylation requires inhibition of multiple PATs (Roth et al., 2006). Calflagins, by contrast, appear to be modified by a single PAT, as indicated by the inability of other enzymes to compensate upon TbPAT7 knockdown. Elucidation of the subcellular distribution of the *T. brucei* PATs, combined with in vitro or bacterial reconstitution experiments, could reveal whether the inability of other PATs to compensate for loss of TbPAT7 results from a lack of activity of other enzymes for calflagins, or sequestration in distinct intracellular sites. Related to this issue is whether pellicular membrane calflagin, which is mislocalized by TbPAT7 RNAi, might be able to be palmitoylated and re-localize to the flagellum upon tetracycline washout, or whether it would remain inaccessible to PAT7. The in vivo specificity of calflagin palmitoylation by TbPAT7 makes this an optimal system for further investigations of the enzymology and molecular basis of PAT-substrate recognition and activity.

## Materials and Methods

### Parasites and antibodies

All *T. brucei* strains described in this study are derivatives of the procyclic 29-13 strain, a 427 strain engineered to co-express bacteriophage T7 RNA polymerase and tet repressor (Wirtz et al., 1999). Antibodies were obtained from the following sources: anti-procyclin (IB 1:1000) from Cedarlane Laboratories (Ontario, Canada); rabbit anti-myc (IF 1:100) from Rockland Immunochemicals (Gilbertsville, PA); mouse monoclonal anti-myc 9E10 supernatant (IB 1:10) and anti-tubulin E7 ascites (IB 1:1000) from the Developmental Studies Hybridoma Bank (Iowa City, Iowa); anti-CAP5.5 (IB 1:500), anti-ROD-1 (IF neat) (Woods et al., 1989) and anti-WCB (IB 1:1000) kindly provided by Keith Gull (Oxford University, Oxford, UK); anti-Hsp70 (IB 1:5000) generated in our laboratory and described previously (Olson et al., 1994); and anti-BiP (IB 1:1000) kindly provided by James D. Bangs (University of Wisconsin, Madison, WI). Calflagin-specific mouse antiserum (IB 1:1000, IF 1:1000) was generated in our laboratory (Tyler et al., 2008).

### Plasmids and transgenic parasite engineering

All RNAi cell lines were created by cloning of the target sequence into the pZJM vector (Wang et al., 2000). Genes predicted to encode proteins with the DHHC-CRD motif (Pfam ID: PF01529) were identified in the *T. brucei* genome database (www.genedb.org). A 400-600 bp region of each gene was selected on the basis of its lack of similarity to other genes using the RNAit software (Redmond et al., 2003). Oligonucleotides (Integrated DNA Technologies, Coralville, IA) were designed to amplify this target region and provide flanking *Hin*dIII and *Xho*I sites. Template DNA was purified as described previously (Medina-Acosta and Cross, 1993) and PCR was conducted with *Taq* polymerase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The amplicon was gel purified (Qiagen, Gaithersberg, MD) and ligated into pCR-2.1-TOPO (Invitrogen). The insert was digested out of this plasmid and ligated into pZJM for the generation of the RNAi construct.

To express epitope-tagged parasite proteins, primers were designed to amplify the desired ORF and provide flanking 5′ *Hin*dIII and 3′ *Xba*I sites. Amplicons were gel purified and subcloned into pCR-BluntII-TOPO before digestion and ligation into pLEW79-Myc, which was generated by the removal of the NOG1 insert from the

plasmid pLEW79-NOG1-Myc (the kind gift of Marilyn Parsons) with *Hin*dIII and *Xba*I. Site-directed mutagenesis of the N terminus was performed using 40-50 bp, gel-purified primers (Integrated DNA Technologies, Coralville, IA) containing the desired mutation and the QuikChangeII kit (Strategene, La Jolla, CA).

To generate transgenic parasites, pZJM and pLEW79-Myc plasmids were linearized by digestion with *Not*I and 10-100 μg of DNA in 100 μl of sterile water were mixed with $2.25\times10^7$ parasites in 450 μl of electroporation medium (120 mM $KCl_2$, 0.15 mM $CaCl_2$, 9.2 mM $K_2HPO_4$, 25 mM HEPES, 2 mM EDTA, 4.75 mM $MgCl_2$, 69 mM sucrose, pH 7.6). Samples were transferred to a 0.4 cm gap cuvette, electroporated twice (1.4 kV, 25 μF) and collected in 5 ml of fresh SDM-79. Drug selection was initiated 6-16 hours post-transfection. All primer and plasmid sequences are available from the authors upon request.

## Microscopy

Parasites were centrifuged for 5-7 minutes at 1000 ***g***, washed twice with phosphate-buffered saline (PBS), and resuspended in 4% paraformaldehyde. Fixed parasites were settled onto poly-L-lysine-coated slides for 30 minutes (all incubations at room temperature), quenched with 50 mM glycine in PBS for 15 minutes and washed with PBS for 30 minutes, exchanging wash buffer approximately every 5 minutes. Cells were then permeabilized with 0.2% Triton-X-100 in PBS for 15 minutes and incubated in blocking buffer [2% normal goat serum, 1% bovine serum albumin (BSA) in PBS] for 30 minutes. Cells were incubated with primary antibodies diluted into blocking buffer for 2-3 hours and washed with PBS over 30-60 minutes with replacement of wash buffer every 5-10 minutes. Cells were incubated with AlexaFluor secondary antibodies diluted 1:400 in blocking buffer for 1 hour and washed as after primary incubation. Cells were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 10-20 seconds, washed for 2 minutes with ultrapure water and mounted with Gelvatol containing 100 mg/ml 1,4-diazabicyclo[2.2.2]octane. Imaging was performed on a Leica DMIRE2 microscope with a 100× objective and a Zeiss AxioImager microscope with a 63× objective. Deconvolution and image analysis was performed with OpenLab (Leica, Wetzlar, Germany), Volocity (Improvision. Seattle, WA), and Axiovision (Zeiss, Thornwood, NY) software.

## Lipid raft analysis

To test for association with detergent-resistant membranes, procyclic stage parasites were resuspended at $1\times10^8$ cells/ml in 1% Triton X-100 in PBS, pre-equilibrated to 4°C or 37°C. Samples were incubated for 10 minutes at either temperature and centrifuged for 7 minutes at 1000 ***g***. The supernatant was removed and transferred to new tube, and an equal volume of 1% Triton X-100/PBS was added to the remaining pellets. The samples were centrifuged again for 7 minutes at 1000 ***g***. Supernatant and pellet fractions were then divided and added to 2× Laemmli sample buffer, boiled for 5 minutes, and analyzed by SDS-PAGE and immunoblotting. Lipid raft association was also tested by flotation of lystate from $2\times10^8$ parasites through a discontinuous Optiprep density gradient as previously described (Fridberg et al., 2008). A total of 12.5 μl from each 1 ml fraction were analyzed by SDS-PAGE and immunoblotting.

## $^3$H-myristate labeling

Myristoylation was assayed by incorporation of $^3$H-myristic acid. For induction of expression of epitope-tagged proteins, 1 μg/ml tetracycline was added 2 hours prior to labeling. A total of $3\times10^7$ parasites were harvested from mid-log cultures, washed once in labeling medium (SDM-79 containing 7.5 mg/ml hemin and 0.5 mg/ml transferrin) and resuspended in 5 ml of labeling medium. Cells were then incubated for 1 hour at 30°C to starve cells of exogenous lipid. Radiolabeled $^3$H-myristic acid (Pierce, Rockford, IL) was concentrated under a stream of nitrogen until the final ethanol concentration during labeling was to be <0.2%. A volume corresponding to 200 μCi of label was then mixed with 14 μl of 1.8% w/v delipidated BSA and added to the parasite culture. After 3 hours of labeling, cells were centrifuged, washed three times in PBS to remove residual label and lysates were harvested in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8.0)]. For immunoprecipitation, 100 μl 9E10 anti-myc monoclonal supernatant were combined with 50 μl Gammabind G Sepharose beads and 1 ml RIPA buffer, and rotated for 2 hours at room temperature. Antibody-bead complexes were washed five times in RIPA buffer before addition of lysates and incubation with rotation overnight at 4°C. Beads were washed five times with RIPA and proteins were eluted by boiling in 100 μl 2× Laemmli sample buffer for 5 minutes. Eluates were fractionated by SDS-PAGE (25 μl per lane) and transferred to nitrocellulose membranes, which were then washed three times with water and treated with Amplify (Amersham, Arlington Heights, IL) for 30 minutes. Membranes were dried and exposed to film using the Transcreen LE (Kodak, Rochester, NY) at –70°C for 3 days before film development.

## Acyl-biotin exchange chemistry

To detect protein palmitoylation, we adapted a protocol developed for yeast (Wan et al., 2007). Briefly, $1\times10^{10}$ parasites were pelleted and washed twice with PBS, then resuspended in 20.75 ml of lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA) supplemented with protease inhibitor mix (Roche, Indianapolis, IN) and 10 mM N-ethylmaleimide (NEM, Sigma, St Louis, MO). Parasites were then lysed by the addition of 4.25 ml of 10% Triton X-100 (Anatrace, Maumee, OH) to give a final concentration of 1.7%. Suspensions were incubated for 1 hour at 4°C with end-over-end rotation. Intact cells and nuclei were pelleted by low speed centrifugation. Supernatants were split into 3 ml aliquots, transferred to 30 ml Corex centrifuge tubes, and subjected to chloroform-methanol precipitation (Wessel and Flugge, 1984). To each 3 ml sample was added 12 ml methanol, 4.5 ml chloroform and 9 ml of water. The tubes were then vortexed and centrifuged at 6000 ***g*** for 20 minutes, the upper phase was aspirated and 9 ml of methanol was added. The tubes were again vortexed and spun at 6000 ***g*** for 10 minutes, the supernatant aspirated and the protein pellets allowed to air dry for 3-5 minutes before resuspension in 600 μl of solubilization buffer (50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 4% SDS) supplemented with 10 mM NEM. To facilitate protein resuspension, the samples were incubated at 37°C for 10 minutes with occasional vortexing. Samples were then diluted with 2.4 ml of lysis buffer containing 0.2% Triton X-100 and protease inhibitors. Blocking proceed overnight at 4°C with rotation. The next day, NEM was removed from the samples by three sequential rounds of chloroform-methanol precipitation, as described above, with pellets after the first and second precipitation resuspended in 750 μl of solubilization buffer and diluted with 2.25 ml of lysis buffer containing 0.2% Triton X-100. Following the third precipitation, pellets were resuspended in 600 μl of solubilization buffer, pooled and split into equal volumes for experimental and control conditions. To one 600 μl sample was added 2.4 ml hydroxylamine-label reagent (200 μM biotin-HPDP, 700 mM hydroxylamine, pH 7.4, 5 mM EDTA, 0.2% Triton X-100, protease inhibitors). To the other was added 2.4 ml Tris-label reagent (200 μM biotin-HPDP, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.2% Triton X-100, protease inhibitors). Samples were incubated at room temperature for 1 hour with end-over-end rotation. The reactions were stopped and the labeling reagent was removed by three sequential rounds of chloroform-methanol precipitation. Resuspension following the first two precipitations was performed as for NEM removal. Following the final precipitation, pellets were resuspended in 150 μl of 2% SDS buffer (50 mM Tris-Cl, pH 7.4, 5 mM EDTA, 2% SDS) and 30 μl was collected to represent the input fraction. To the remaining 120 μl was added 2.28 ml of lysis buffer to dilute out SDS that would interfere with the streptavidin-biotin interaction. Samples were then added batchwise in 1.2 ml aliquots to tubes containing 40 μl streptavidin-agarose beads that had been pre-washed with pulldown wash buffer (lysis buffer containing 0.2% Triton X-100 and 0.1% SDS). After 90 minutes of rotation at room temperature, beads were washed five times with pulldown wash buffer. Samples were eluted by incubating beads in 22.5 μl pulldown elution buffer (lysis buffer containing 0.2% Triton X-100, 0.1% SDS, 1% β-mercaptoethanol). Input and elution fractions were mixed with 2× Laemmli sample buffer, boiled for 5 minutes and analyzed by immunoblotting of ~$10^6$ cell equivalents loaded per lane.

## Statistical analysis

Confidence intervals of proportions were calculated by the ‘score method incorporating continuity correction’ described by Newcombe (Newcombe, 1998).

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