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Exploring protein myristoylation in *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is an important human and veterinary pathogen and the causative agent of toxoplasmosis, a potentially severe disease especially in immunocompromised or congenitally infected humans. Current therapeutic compounds are not well-tolerated, present increasing resistance, limited efficacy and require long periods of treatment. On this context, searching for new therapeutic targets is crucial to drug discovery. In this sense, recent works suggest that N-myristoyltransferase (NMT), the enzyme responsible for protein myristoylation that is essential in some parasites, could be the target of new anti-parasitic compounds. However, up to date there is no information on NMT and the extent of this modification in *T. gondii*. In this work, we decided to explore *T. gondii* genome in search of elements related with the N-myristoylation process. By a bioinformatics approach it was possible to identify a putative *T. gondii* NMT (TgNMT). This enzyme that is homologous to other parasitic NMTs, presents activity *in vitro,* is expressed in both intra- and extracellular parasites and interacts with predicted TgNMT substrates. Additionally, NMT activity seems to be important for the lytic cycle of *Toxoplasma gondii*. In parallel, an *in silico* myristoylome predicts 157 proteins to be affected by this modification. Myristoylated proteins would be affecting several metabolic functions with some of them being critical for the life cycle of this parasite. Together, these data indicate that TgNMT could be an interesting target of intervention for the treatment of toxoplasmosis.

Graphical Abstract

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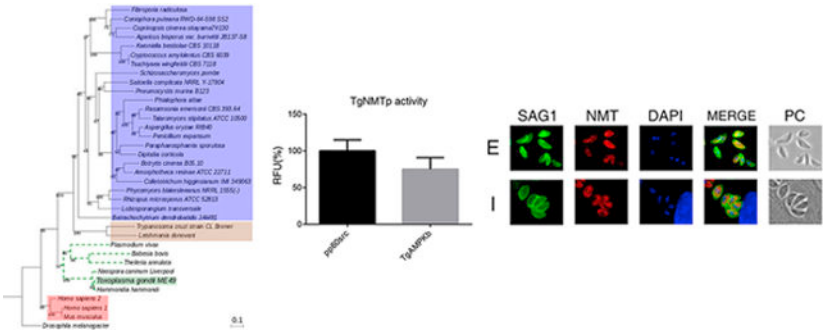

Keywords

Toxoplasma gondii; protein myristoylation; N-myristoyltransferase; myristoylome; calcium homeostasis

1. INTRODUCTION

N-myristoylation is a lipid modification that is estimated to affect between 1–4% of all proteins in higher eukaryotes (Martinez, et al., 2008). This modification is characterized by the covalent attachment of the fourteen-carbon fatty acid myristate to an essential N-terminal glycine residue via an amide bond after the removal of the initiator methionine. The N-myristoylation process is catalyzed by myristoyl-CoA:protein N-myristoyltransferase (NMT), a member of the GCN5-related N-acetyltransferases (GNAT) superfamily of proteins (Farazi, et al., 2001). The consensus sequence recognized by *N*-myristoyltransferase (NMT) is M-G-X-X-X-S/T/C where X represents most amino acids except for Proline (P), aromatic or charged residues in position X_3 and preferentially a lysine or arginine residue at position 7 and/or 8 (Farazi, et al., 2001). This protein modification plays a direct role in cellular signaling and subcellular targeting by promoting membrane binding and modulating protein-protein interactions (Wright, et al., 2010). Currently, our knowledge of protein myristoylation has expanded to understand that the mechanism of protein myristoylation is well conserved in eukaryotic organisms including relevant human pathogens such as *Plasmodium spp*. and *t*rypanosomatids like *Trypanosma brucei*, *Trypanosoma cruzi* and *Leishmania spp*. (reviewed in (Martin, et al., 2011)). Studies based on NMT inhibition in pathogens confirm that N-myristoylation is relevant for proliferation of these parasites since myristoylated proteins participate in cell replication, migration and host-cell invasion (Beck, et al., 2010, Frenal, et al., 2010). Furthermore, and contrary to what was observed for higher eukaryotes, parasites express only one NMT (Martin, et al., 2011, Wright, et al., 2014). In this manner, this acyltransferase is considered a good potential target for the development of new treatments for these and other pathogens (Wright, et al., 2014).

Toxoplasma gondii is a human and veterinary pathogen member of *Apicomplexa* phylum (Montoya and Liesenfeld, 2004, Tenter, et al., 2000). This protozoan is considered of great relevance for the human health since its infection produces toxoplasmosis, an infectious disease that affects about one third of the global population with high morbidity and mortality rates especially in South and Central America and Continental Europe (Bangoura,

et al., 2011, Furtado, et al., 2011, Meerburg and Kijlstra, 2009). Currently, the most effective *T. gondii* treatment is a combination of pyrimethamine and sulfadiazine, although both drugs are not well-tolerated and present increasing resistance (Montoya and Liesenfeld, 2004, Petersen, 2007). Due to this, is that there is an urgent need of finding new targets of intervention. Given the fact that parasites express only one NMT that seems to be essential for their viability is that TgNMT appears as a good target of intervention. However, the scope and role of protein myristoylation in *T. gondii* has not been addressed yet.

Up to present, there are no reports on the existence of any N-myristoyltransferase in *T. gondii* or on the importance and extent of protein myristoylation on this parasite. As such, in this work we screened the Toxoplasma database (www.toxodb.org) and found a single putative gene product that could act as an N-myristoyltransferase (TgNMT). We confirmed that this gene product presents myristoyltransferase activity *in vitro* and is expressed in the cytoplasm of intra- and extracellular parasites. Furthermore, NMT activity would be required for a normal lytic cycle since inhibition of myristoylation with 2-hydroxymyristic acid results in a reduced total lysis area when compared to controls. Finally, an *in silico* screen of the database showed that *T. gondii* myristoylome would encompass a total of 157 potential myristoylable proteins with some of them found to interact with TgNMT. Interestingly, other putative myristoylated proteins are associated to calcium regulation, which is critical for *T. gondii* life-cycle and thus, supporting the effect observed on the lytic cycle. Together, these results position TgNMT as an attractive drug target of intervention for the treatment of toxoplasmosis and at the same time suggest a complex interplay between protein myristoylation and calcium homeostasis.

2. MATERIALS AND METHODS

2.1 Antibodies and reagents

Specialized and common reagents were from Sigma, unless specified. ECL Plus was from GE Biosciences. Alexa-conjugated secondary antibodies were from Molecular Probes. Tissue culture reagents were from Invitrogen.

2.2 Toxoplasma *and host-cell cultures*

T. gondii tachyzoites of the RH Δhxgprt strain (Donald, et al., 1996) were used throughout the study. Parasites were maintained by serial passage on confluent monolayers of human foreskin fibroblasts (HFFs) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% v/v bovine serum albumin (BSA), 100 i.u. (international units) /ml penicillin and 100 μg/ml streptomycin. When required, freshly lysed tachyzoites were purified using a 3 μm polycarbonate filter.

2.3 Toxoplasma gondii *database analysis*

The search for putative N-myristoyltransferase gene products in the *T. gondii* genome was performed using the TGME49 strain with the interpro domain tool (ToxoDB v28), using the pfam (www.pfam.org) database as default. The screening was performed using the pfam 01233 towards the N-terminal end of the protein and pfam 02799 towards the C-terminal end.

For the generation of the *in silico* myristoylome, we used the ToxoDB v28 web page (www.toxodb.org). The TGME49 strain was screened using the "similarity/pattern tool", utilizing a combination of the conserved myristoylation pattern and its exceptions (Martin, et al., 2011). The results were analyzed with the gene ontology (GO) analysis tool for molecular function, biological process and cellular component with a p-value of 0.05. The p-value was calculated with the Fisher's exact test. Proteins that were experimentally determined to be palmitoylated in *T. gondii* are marked with (+). Polybasic domains found in the first 25 amino acids were determined by the ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/) and lipidic modifications excluding myristoylation were determined by GPS-Lipid v1.0 program (Xie, et al., 2016).

2.4 Phylogenetic analysis of the predicted NMT

Reference sequences related to TGME49_209160 amino acid sequences were obtained by a BLASTp search on Apicomplexa (taxid:5794), *Homo sapiens* (taxid:9606), Trypanosomatidae (taxid:5654) and Fungi (taxid:4751) databases at NCBI domain (Madden, 2013). Reference amino acid sequences from *Trypanosoma brucei*, *Mus musculus* and *Droshopila melanogaster* were added manually; the final set of amino acid sequences used is listed in Table S1. A total of 36 sequences were aligned using MAFFT (Multiple Alignment using Fast Fourier Transform) under default settings (Katoh and Standley, 2013). A multiple alignment was subsequently analyzed and cleaned using the trimAl software in automated 1 mode (Capella-Gutierrez, et al., 2009). The trimmed alignments are shown in Figure S1. The tree was constructed by a maximum likelihood algorithm with RaxML software using the Gamma Blosum62 Protein model and rapid bootstrapping method (Stamatakis, 2014). Artwork and annotation was done by the interactive Tree Of Life (iTOL) program v 4.3 (Letunic and Bork, 2016).

2.5 Enzymatic activity determination

NMT activity determination was carried out as described by Goncalves and coworkers (Goncalves, et al., 2012). Briefly, 6.8 nM of purified TgNMT (*E. coli* expressed recombinant protein obtained as described in supplementary material) was incubated with the reaction mixture in a final volume of 220 ml. This mixture contained 20 mM Na_2HPO_4/NaH_2PO_4 pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA and 0.1 % Triton X-100 (v/v), 15 mM myristoyl-CoA and 8 mM CPM (7-diethylamino-3-(4-maleimido-phenyl)-4-methylcoumarin; a fluorescent probe). The reaction was initiated by the addition of 12 ml of a peptide containing-solution (final concentration 15 mM). The peptide substrate used corresponded to the N-terminal end of either pp60src (amino acid sequence: NH2-GSNKSKPK-CO-NH2 (King and Sharma, 1994)) or the putative *T. gondii* substrate TGME49_268960 (amino acid sequence: NH2-GSQTSNSR-CO-NH2). Both peptides were synthesized at GenScript, USA. The reaction was incubated at 25°C for 30 minutes a nd the fluorescence emission at 470 nm was determined (excitation at 384). The NMT activity is expressed as percentage of the relative fluorescence units obtained when pp60src is used as substrate. Determinations were carried out in 96 well black bottom microplates (FLUOTRACTM 200, Greiner) using Multi modal Synergy H1 (BioTek).

2.6 Indirect immunofluorescence studies

All the steps were carried out at room temperature. For intracellular parasites, 8×10^5 freshly lyzed tachyzoytes were allowed to invade a confluent monolayer of HFF cells. After 1-hour incubation at 37°C, media was discarded and the cells were washed twice with PBS. Cells were fixed with formaldehyde 4% v/v in PBS for 30 minutes followed by a 1 minute wash in PBS. Then, cells were permeabilized with 0.3% v/v Triton X-100 in PBS for 20 minutes and blocked with 3% w/v BSA in PBS for 30 minutes. After this, primary antibodies were diluted in 3% w/v BSA in PBS: mouse anti-SAG1:1/200 (kindly provided by Dr Clemente, INTECH, Argentina; (Albarracin, et al., 2015)) and rabbit anti-TgNMT: 1/1,000 (Supplemental material) and then incubated for 60 minutes followed by extensive washes. Secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG (1/1,000) and Alexa Fluor 594- conjugated goat anti-rabbit (1/1,000; Invitrogen). Finally, samples were mounted with Fluoromont (Abcam). When extracellular parasites were used (2×10^6), they were fixed on microscopy slides with formaldehyde 4% v/v in PBS for 30 minutes followed by a 1 minute wash in PBS. Then, the same protocol mentioned above was followed. Parasites were imaged using an Eclipse E600 microscope (Nikon).

2.7 Plaque assay

Freshly lysed purified parasites (200 parasites) were used to infect HFF cell monolayers seeded in 24-well plates in presence of 0 (vehicle), 6.25, 12.5, 25, 50 or 100 mM 2-hydroxy myristate (2HMA) in DMSO (final concentration 0.1% v/v). Then, infected cells were incubated for 7 days at 37°C, 5 % CO_2 without movement. Cells were then fixed with 70% ethanol and stained with 0.1% crystal violet. Plaques were scanned using an Epson scanner. For plaque count and size, plaque images were opened with the ImageJ software, converted to grayscale and a threshold adjustment was performed to highlight plaque limits. The plaques area was approximated in pixels by the measure tool. Experiments were repeated twice independently with two replicates each time.

2.8 Cell proliferation assay

Monolayers of hTERT cells were grown in 96-well flat-bottom tissue culture microtiter plates at 37°C 5% CO2 and incubated with different concentrations of 2-hydroxy myristic acid (2HMA), supplied at a final concentration of 0.5 % v/v DMSO in complete DMEM. After 6 days, cells were washed and 0.5 mg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] added. Plates were kept 4 hours at 37°C, then cells disrupted and formazan product solubilized in DMSO 100 % and absorbance at 540 nm recorded using a 96-well plate reader (Synergy Mx BioTek). Values are presented as percentages relative to cells incubated without 2HMA (100% survival).

2.9 Generation of TgNMT interactome

Immunoprecipitation was performed as previously described by Adams *et al.* (Adams, 2002) with minor modifications. Briefly, freshly lysed tachyzoites (2×10^8) were resupended in 2 ml lysis buffer (Tris-HCl 50 mM, NaCl 120 mM and 0.5% v/v Nonidet P-40, pH8). Lysates were obtained by sonication and cellular debris was discarded by centrifugation for 15 minutes at 10,000 xg, 4°C. Then, the sample was divided in 2 halves adding in each half

either 10 ml anti-TgNMT or 10 ml pre-immune sera. After 16–18 hour incubation at 4°C, protein A/G sepharose (previously equilibrated in lysis buffer) was added to both samples and incubation continued for another hour at 4°C. Then, immunocomplexes were was hed 5 times in lysis buffer and finally resuspended in 50 ml lysis buffer. Bound proteins were eluted by heating samples for 5 minutes at 80°C. An aliquot of the eluates (10% of total volume) were analyzed by SDS-PAGE and the rest acetone-precipitated to be analyzed by MudPIT.

2.10 MudPIT analysis of immunoprecipitates

Protein pellets were washed 2 times with 350 ml ice-cold acetone. Air-dried pellets were dissolved in 8 M urea/ 100 mM Tris-HCl pH 8.5. Proteins were reduced with 1 M Tris (2-carboxyethyl) phosphine hydrochloride and alkylated with 500 mM 2-chloroacetamide. Proteins were digested for 18 hr at 37 °C in 2 M urea, 100 mM Tris-HCl pH 8.5, 1 mM $CaCl_2$ with 2 mg trypsin (Promega, Madison, WI). Digest was stopped with formic acid, 5% final concentration. Debris was removed by centrifugation, 30 minutes 18,000 xg.

A MudPIT microcolumn (Wolters, et al., 2001) was prepared by first creating a Kasil frit at one end of an undeactivated 250 mm ID/360 mm OD capillary (Agilent Technologies, Inc., Santa Clara, CA). The Kasil frit was prepared by briefly dipping a 20 – 30 cm capillary in well-mixed 300 ml Kasil 1624 (PQ Corporation, Malvern, PA) and 100 ml formamide, curing at 100°C for 4 hrs, and cutting the frit to ~2 mm in length. Strong cation exchange particles (SCX Partisphere, 5 mm dia., 125 Å pores, Phenomenex, Torrance, CA) were packed in-house from particle slurries in methanol 2.5 cm. An additional 2.5 cm reversed phase particles (C18 Aqua, 3 μm dia., 125 Å pores, Phenomenex) were then similarly packed into the capillary using the same method as SCX loading, to create a biphasic column. An analytical RPLC column was generated by pulling a 100 mm ID/360 mm OD capillary (Polymicro Technologies, Inc, Phoenix, AZ) to 5 mm ID tip. Reversed phase particles (Aqua C18, 3 mm dia., 125 Å pores, Phenomenex, Torrance, CA) were packed directly into the pulled column at 800 psi until 12 cm long. The MudPIT microcolumn was connected to an analytical column using a zero-dead volume union (Upchurch Scientific (IDEX Health & Science), P-720–01, Oak Harbor, WA).

LC-MS/MS analysis was performed using an Agilent Technologies 1200 HPLC pump and a Thermo LTQ-Orbitrap XL using an in-house built electrospray stage. MudPIT experiments were performed with steps of 0% buffer C, 20% buffer C, 50% buffer C, 100% C, and 90/10 % buffer C/B, being run for 4 minutes at the beginning of each gradient of buffer B. Electrospray was performed directly from the analytical column by applying the ESI voltage at a tee (150 mm ID, Upchurch Scientific; (Wolters, et al., 2001)). Electrospray directly from the LC column was done at 2.5 kV with an inlet capillary temperature of 275 OC. Data-dependent acquisition of MS/MS spectra with the LTQ –Orbitrap XL were performed with the following settings: MS/MS on the 10 most intense ions per precursor scan, 1 microscan, reject charge state 1; dynamic exclusion repeat count, 1, repeat duration, 30 second; exclusion list size 500; and exclusion duration, 60 second.

Protein and peptide identification and protein quantitation were done with Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, Inc., San Diego, CA. http://

www.integratedproteomics.com/). Tandem mass spectra were extracted from raw files using RawConverter and were searched against Uniprot Toxoplasma protein database with reversed sequences using ProLuCID (He, et al., 2015, Peng, et al., 2003). The search space included all fully-tryptic peptide candidates. Carbamidomethylation (+57.02146) of cysteine was considered as a static modification. Peptide candidates were filtered using DTASelect (Tabb, et al., 2002).

3. RESULTS

3.1 Toxoplasma gondii *encodes for a single putative N-myristoyltransferase*

In order to identify sequences coding for proteins with N-myristoyltransferase activity in *Toxoplasma gondii*, a search using the TOXODB database was performed (www.toxodb.org; (Gajria, et al., 2008)). The screening was carried out using the PFAM consensus sequences typical of the NMT family of proteins (the PF01233, myristoyl-CoA binding region and the PF02799 substrate protein-binding region; (Weston, et al., 1998)). Only one protein, product of the TGME49_209160 gene was retrieved (E value = $9.0E^{-75}$). This suggests that only one copy of NMT is present in *T. gondii* (paralog count = 0), as is observed in many lower eukaryotes.

A phylogenetic analysis was generated with the deduced amino acid sequence of TGME49_209160 and myristoyl-CoA:protein N-myristoyltransferases (NMTs) from different eukaryotic organisms including parasites, fungi and mammals. Figure 1 shows that the predicted *T. gondii* NMT (TgNMTp) is in the same evolutionary branch as other members of the phylum Apicomplexa while sequences related to trypanosomatid parasites are grouped in a distant clade where sequences of fungi organisms are the most represented. This analysis highlights that humans NMTs (HsNMTs) and *Mus musculus* sequences are grouped in a distant clade from TgNMTp. This result suggests that there are significant evolutionary differences between these NMTs and that this difference could be of importance in attempts to design specific inhibitors.

Multiple amino acid sequence alignments shows highly conserved regions between putative *T. gondii* NMT and NMTs described in other species (Figure S2). Notably, the residues that integrate the two PROSITE signature sequences of NMT enzymes (PS00975 at the N-terminal domain and PS0076 at the C-terminal domain), together with the presence of key amino acids for the catalysis, strongly suggest that TGME49_209160 codes for a functional myristoyl-CoA: protein N-myristoyltransferase.

3.2 TgNMTp is a functional N-myristoyltransferase that expresses throughout the lytic cycle

In order to determine if TgNMTp presents N-myristoyltransferase activity, TGME49_209160 coding sequence was successfully expressed in *E. coli* and the protein purified (Supplemental Material). TgNMTp, obtained with high degree of purity and homogeneity, was used for the determination of enzymatic activity and to generate specific antibodies (Supplemental material). The N-myristoyltransferase activity was assayed according to Goncalves *et al.* using two octapeptides as a substrates (Goncalves, et al.,

2012). The first one, G-S-N-K-S-K-P-K-NH_2, is a derivative of the N-terminal sequence of *H. sapiens* proto-oncogene tyrosine kinase pp60src. This peptide was used as a standard substrate since humans and *S. cerevisiae* NMTs were able to transfer the myristate moiety to their amino terminal Gly residue (Rocque, et al., 1993). The second octapeptide (G-S-Q-T-S-N-S-R-NH_2) was synthesized based on the results obtained in our *in silico* myristoylome (Table S3). This putative myristoylable peptide corresponds to the N-terminal end of *T. gondii* TgAMPK β subunit protein (accession number ToxoDB: TGME49_268960). Figure 2A shows that TgNMTp presented myristoyl transferase activity towards both substrates.

To determine TgNMT subcellular localization and expression throughout the lytic cycle, indirect immunofluorescence study (IFI) on intra- and extracellular tachyzoites was carried out. As other NMTs, *T. gondii* N-myristoyltransferase is localized to the cytosol of both on intra- and extracellular parasites (Figure 2B).

3.3 *TgNMT activity is important for in* T. gondii *lytic cycle*

Based on our previous observations, we decided to assess whether TgNMT activity is required during tachyzoite lytic cycle. In order to do this, we incubated infected HFF monolayers with increasing concentrations of 2-hydroxymyristic acid (2HMA). This analog of myristic acid becomes metabolically activated in cells to form 2-hydroxymyristoyl-CoA, a potent inhibitor of NMT (Paige, et al., 1990). Figure 3 shows that when infected cells are treated with 2HMA at low concentrations no effect was observed. However, at the highest concentration tested (50 mM) a decrease of approximately 50% of the total area lysed by the tachyzoites was observed (Figure 3). On the other hand, no effect in host-cell viability was observed under our experimental conditions. These data suggest that NMT activity is important for optimal lytic cycle under tissue-culture conditions.

3.4 TgNMT interacts with various proteins

In order to obtain information on proteins interacting with TgNMT, we performed immunoprecipitations using the anti-TgNMT serum generated in our laboratory (Supplemental material) followed by MudPIT analysis for subsequent identification by spectral count analysis. Spectral Counting (SpC) is a viable strategy for free labeling proteomics, where the number of spectra corresponding to the peptides of a protein is used as a measure of its abundance (Liu, et al., 2004). Our crude results show a total of 433 putative interacting proteins and we calculated the enrichment of every protein based on fold change in SpC detection. Thus, proteins having a fold change value equal or greater than two were considered as enriched. In this manner we obtained a total of 66 putative interacting proteins (Figure 4 and Table S2, highlighted in red).

Since protein myristoylation is in general a co-translational modification, it is not surprising to find ribosomal proteins in our analysis or relevant proteins for the translation process (Figure 4C, bars with asterisk). Ribosomal proteins detected in our analysis allow inferring an interaction with TgNMT as it was observed in other eukaryotes (Farazi, et al., 2001).

3.5 T. gondii *myristoylome would encompass several proteins with varied functions*

For a given protein to be recognized and modified by NMT, it should contain at its N-terminus the following amino acid consensus sequence: $M_1-G_2-X_3-X_4-X_5-(S/T/C)_6$, where X represents most amino acids except for Proline (P), aromatic or charged residues in position X_3. It is important to highlight that other amino acids can be tolerated at position X_6 (Alanine,A; Glycine,G) and even a Lysine(L) residue at position 7 can reduce the stringency for certain amino acids at position 2 (revised in (Martin, et al., 2011)). With the aim of determining the extent of myristoylation in *T. gondii*, an *in silico* screening over the TGME49 strain gene products was performed. Using the tool for protein motif pattern identification, and the consensus amino acid sequence with the exceptions described above, we found 181 gene products that could be myristoylated. Since protein myristoylation occurs mainly on cytoplasmic proteins, we discarded sequences containing predicted signal peptides, thus obtaining a total of 157 gene products (Table 1 and Table S3 for the complete list of proteins). This represents 1.77% of the total *T. gondii* ME49 strain proteome which is in accordance with other reports stating that protein myristoylation covers between 1–4% of all proteins on a given proteome (Martinez, et al., 2008).

A Gene Ontology analysis (GO) of the theoretical myristoylome suggests a possible role of the gene products in the life cycle of the parasite. The most enriched products participate in processes such as protein phosphorylation (GO:0006468; Table S4) and lipopolysaccharide biosynthetic process (GO:0009103). Twenty three gene products share the five most enriched biological processes in our *in silico* myristoylome ($-\log_{10}= 4.37$; Figure 5, Biological Process), many of which are kinases, principally calcium dependent protein kinases (CDPKs). Additionally our molecular function analysis show that these twenty three products are associated with kinase activity being calmodulin-dependent protein kinase activity the most enriched function (GO:0004683,$-\log_{10}= 6.15$; Figure 5, Molecular Function). These gene products are annotated to contain at least one kinase domain, where CDPKs are again the kinase family most represented among other gene products with confirmed or predicted kinase activity (Table S5). CDPKs are proposed as good drug target candidates in *Plasmodium spp.* and *Toxoplasma spp* (Tate, et al., 2014), are predicted to be myristoylated and could function as calcium –myristoyl switches. Furthermore, CDPKs and some of these twenty three proteins showed a phenotype when the corresponding genes were deleted (Sidik, et al., 2016).

A cellular component GO analysis revels that myristoylated proteins are widely distributed within the cell. This is attributed mainly to gene products that present kinase activity (the most represented in the myristoylome) which are not associated to any subcellular compartment (Figure 5, Cellular component). The vast representation of kinases in our *in silico* analysis could open a new study in TgNMT activity and localization regulation by phosphorylation, as TgNMT is confirmed to be phosphorylated *in vivo* by the *T. gondii* phosphoproteome (Treeck, et al., 2011). This analysis suggests a relevant role of protein myristoylation in *T. gondii*, as our results reveal gene products that were confirmed to be essential for the pathogen survival, with an important percentage of these proteins confirmed to be palmitoylated as well. As already shown in previous studies protein myristoylation is essential for subsequent palmitoylation (Resh, 2016). In this manner, myristoylation can

directly and indirectly affect a vast diversity of proteins in *T. gondii* that are confirmed to influence its life cycle.

3.6 *Human and* T. gondii *NMT substrates differ in their N-terminal amino acid occurrence*

Analyzing NMT substrates amino acid sequence could help to understand the specificity for this acyl-transferase. With this in mind, we compared the amino acid composition of HsNMT and putative TgNMT peptide substrates focusing in the amino terminal region. Initial methionine and the conserved glycine at the second position in analyzed sequences were deleted with the aim of highlighting differences between myristoylated sequences. A WebLogo analysis shows that the first six residues are similar in both species being asparagine (N) in position three and serine (S) in position six the most characteristic. A clear difference is shown at position seven were the most representative amino acid in human sequences is alanine (A) while lysine (K) is preferred at this position in *T. gondii* sequences. Amino acids at positions 7 to 18 show high variability, but in-depth analysis over the logo sequences show relevant differences: charged amino acids are preferred in *H. sapiens* sequences (positions 8, 9, 13, 15, 17 and 18), while in *T. gondii* neutral and small amino acids are more frequent (Figure 6).

4. DISCUSSION

Protein N-myristoylation refers to the irreversible covalent attachment of 14-carbon myristic acid onto an N-terminal glycine by N-myristoyltransferases (NMTs, (Farazi, et al., 2001)). This important protein modification plays roles in cellular signaling pathways, mediating subcellular targeting and protein-protein interactions (thoroughly reviewed in (Martin, et al., 2011)). However, there is scarce information on this protein modification in *Toxoplasma gondii*. With this in mind, we decided to explore the extent and roles of protein myristoylation in this parasite.

First we decided to determine whether *T. gondii* expressed a NMT. In order to do this, we performed an *in silico* screening across the *T. gondii* database and found only one gene product that could be *T. gondii* NMT (TgNMTp). The finding of a single putative TgNMT is not unusual, since contrary to what was observed in higher eukaryotes where two NMT isoforms are expressed, parasites express only one NMT which is essential. In fact, single NMTs have been described for *Leishmania major*, *Leishmania donovani*, *Trypanosoma brucei* and *P. falciparum* (Brannigan, et al., 2010, Price, et al., 2003, Wright, et al., 2014). Besides, our phylogenetic analysis showed that this protein presents homology to other apicomplexan NMTs and at the same time highlights that humans NMTs (HsNMTs) and *Mus musculus* sequences are grouped in a distant clade from TgNMTp. This difference between parasitic and their host NMTs could be exploited for the design of specific inhibitors. In fact, *Plasmodium falciparum* NMT is being the target of small molecule inhibitors to treat malaria (Schlott, et al., 2018, Wright, et al., 2014). The presence of conserved amino acids that have been shown to be critical for NMT activity on TgNMTp led us to think that this protein could present enzymatic activity. In line with this, here we showed that TgNMTp presents NMT activity *in vitro* and the typical cytosolic localization (McIlhinney and McGlone, 1996) in intra and extracellular tachyzoites, suggesting that its

expression is needed through the lytic cycle. This was further confirmed when incubation of intracellular parasites with the NMT inhibitor 2HMA showed a decreased total lysed area, suggesting that the lytic cycle was altered. Since protein myristoylation can direct a protein to its site of action influencing its correct functioning, a decrease in host-cell lysis by the parasite can be attributed to myristoylation inhibition of proteins that are important for motility (TgGAP45/70, TgCDPK1), early tachyzoite endodyogeny (TgCDPK7, TgISP2), egress (TgCDPK3) and/or invasion (TgCDPK1, TgGAP45). In this manner the lytic cycle would be deregulated, leading to lower performance of host-cell lysis by *T. gondii*. The effect of 2HMA could be observed at concentrations that did not affect host-cell viability, suggesting a differential affinity between TgNMT and its host counterparts. The lack of effect observed on host-cells could be due to the concentrations of 2HMA used. It is well documented that the highest concentration of 2HMA that produces alteration in myristoylation without having a cytotoxic effect in mammalian cells is 1 mM (Galbiati, et al., 1996, Martin, et al., 2008, Paige, et al., 1990).

Immunoprecipitation assays provided a glimpse of TgNMT interactors. As expected of a co-translational modification, several ribosomal proteins were detected (Figure 4 and Table S2). It was reported that the N-terminal region of all described NMTs interacts with the ribosome during protein translation (Farazi, et al., 2001). Interestingly 12 proteins found to interact with TgNMT were also predicted to be myristoylated by our *in silico* myristoylome and thus, were considered putative TgNMT substrates (Table S6). Only one of the twelve possible substrates was significantly enriched ((fold change: 2.5; ID: TGME49_310420; Figure 4C, bar with arrowhead), while the others present low fold change values (Table S6). Despite this, we detected TgGAP45 (a reported myristoylated protein; (Frenal, et al., 2010)) and three MORN repeat-containing protein, a microneme protein (TgMIC7), a SRS (SAG related sequence) protein, a protein phosphatase 2C-like (reported to be myristoylated in *L. donovani*; (Wright, et al., 2015)) and three uncharacterized proteins. The low enrichment of these gene products could be due either to technical limitations, since immunoprecipitation assays detect proteins that interact with the protein of interest only at the time of the experiment or to the dynamics and distribution of the process, which is linked to translation. In this manner, possible myristoylable substrates are detected as a function of their abundance at this particular stage. As such, future studies of *T. gondii* myristoylome should include other techniques such as metabolic tagging followed by MudPIT for protein identification as already described for *Leishmania donovani* (Wright, et al., 2015).

In order to understand the full extent and role of myristoylation in *Toxoplasma gondii*, we used a bioinformatics approach to generate an *in silico* myristoylome. Our results show that 1.77% of *T. gondii* total proteome could be myristoylated. This is in agreement with repvious reports stating that myristoylation covers 0.5– 3% of all the proteins in a given proteome (Martinez, et al., 2008, Maurer-Stroh, et al., 2002).

Gene ontology analyses show that the most represented protein-function is the calmodulin protein kinase activity; this molecular function is related to our myristoylome by gene products like CDPKs (calcium-dependent protein kinases), ULK kinases and CAM Kinase family. Particularly, CDPKs have been thoroughly studied and documented to play a key role in *T. gondii* life cycle. Thus, CDPKs are being proposed as drug targets for antiparasitic

design (Billker, et al., 2009). These kinases contain consensus motifs for N-myristoylation or palmitoylation, a feature also observed in plant CDPKs, many of which show membrane localization (Dammann, et al., 2003). In fact, mutation of the N-terminal glycine of TgCDPK3 prevents myristoylation which alters the membrane location that is required to carry out its function (Garrison, et al., 2012). Finally, a comparative analysis with the experimental data published by Sidik and coworkers revealed that 66% of the proteins present in our analysis are relevant to the progression of the lytic cycle (Sidik, et al., 2016). Hence, studies on the influence of myristoylation on these proteins would be of great importance. Myristoylomes have been described for parasites including trypanosomatids, *Leishmania donovani* and *Plasmodium falciparum* (Herrera, et al., 2016, Ritzefeld, et al., 2018, Wright, et al., 2016, Wright, et al., 2015). A comparative analysis of these myristoylomes with our *in silico* study reveals some similarities between substrates of *P. falciparum* and *T. gondii* NMTs. Proteins participating in traffic and secretion (ADP-ribosylation factor family proteins, Rab5b), motility (GAP45, CDPK1) and IMC integrity (ISP1–3, GAP45) are found in both myristoylomes (Wright, et al., 2014). Besides, *T. gondii* myristoylome include homologs of proteins described to be myristoylated in *L. donovani* such as protein phosphatase 2C-like, phosphatidylinositol-4-phosphate 5-kinase and the ADP-ribosylation factor family of proteins (Wright, et al., 2015). These comparative results support the strategy used in this work to obtain *T. gondii* myristoylome. It is important to highlight that this myristoylome includes proteins that have already been reported to be myristoylated such as TgISP1–3, TgGAP45 and TgCDPK3 (Beck, et al., 2010, Frenal, et al., 2010, Lourido, et al., 2012). TgGAP45, TgCDPK7 and TgCDPK1 are essential proteins required for the parasite's life-cycle (Frenal, et al., 2010, Lourido, et al., 2010, Morlon-Guyot, et al., 2014). Another essential protein that is included in our list is the enzyme carbamoyl phosphate synthase (CPS), documented to be essential for *T. gondii* virulence (Fox and Bzik, 2002). Additionally 28 genes products detected in this work are confirmed to be palmitoylated as well (Table S3; (Caballero, et al., 2016, Foe, et al., 2015)), including three morn-repeat containing proteins and the membrane occupation and recognition nexus protein 2 (TgMORN2). MORN motifs play critical roles in several proteins with roles in the organization of membranous and cytoskeletal structures while TgMORN2 is a redundant member of the cell assembly process during the replication of the parasite (Gubbels, et al., 2006). Additionally, 63 gene products are listed as hypothetical proteins in the *T. gondii* database and described to be essential (Sidik, et al., 2016), which suggest that the role and extent of protein myristoylation in this parasite is an unexplored field and that deserves to be studied in more detail.

Regarding amino acid occurrence on NMT substrates, it has been described that the first eight N-terminal amino acids are determinant for NMT specificity and selectivity (Castrec, et al., 2018). However, it was also reported that positions in substrate sequence beyond position 8 could have an impact on NMT specificity (Maurer-Stroh, et al., 2002). Here we analyze the amino acid sequences of the putative TgNMT substrates predicted by our *in silico* approach and compare them with host myristoylated substrates. We found that human myristoylated substrates present charged amino acids in positions 7 to 18 more frequently whereas in *T. gondii* neutral amino acids are preferred on these positions. These differences in the amino acidic composition of NMTs substrates in positions beyond the classical

sequence could be useful for the design of specific inhibitors to target parasitic NMTs. This observation could be related with the NMT structure, since it has been documented that a region distal to the active site of the enzyme might accommodate myristoylable peptides (Maurer-Stroh, et al., 2002). Is important to highlight that no crystal structure for TgNMT is available and a structural study of TgNMT could be valuable to understand the differences observed in our study between human and *T. gondii* NMT substrates. Furthermore, a structural analysis of TgNMT could be of great relevance for inhibitor designing.

In summary, our results strongly suggest that myristoylation is a mechanism operating in *T. gondii* with impact on its lytic cycle, particularly in calcium-dependent processes which are essential for growth. As such, is that protein myristoylation could have a central role in *T. gondii* pathogenesis. Furthermore, as observed in other parasites, *T. gondii* expresses only one NMT with apparent differences to its host counterparts. These two observations suggest that TgNMT could be an interesting target for anti-toxoplasmic compunds. Finally, here we provide the first evidence of possible TgNMT substrates, being necessary confirm myristoylation targets by specific protocols such as click-chemistry assays (Yap, et al., 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Toxoplasma gondii* encodes for a single putative N-myristoyltransferase
- A phylogenetic analysis suggests significant differences between TgNMT and HsNMT
- *T. gondii* myristoylome include 157 predicted proteins
- TgNMT activity is important for the lytic cycle

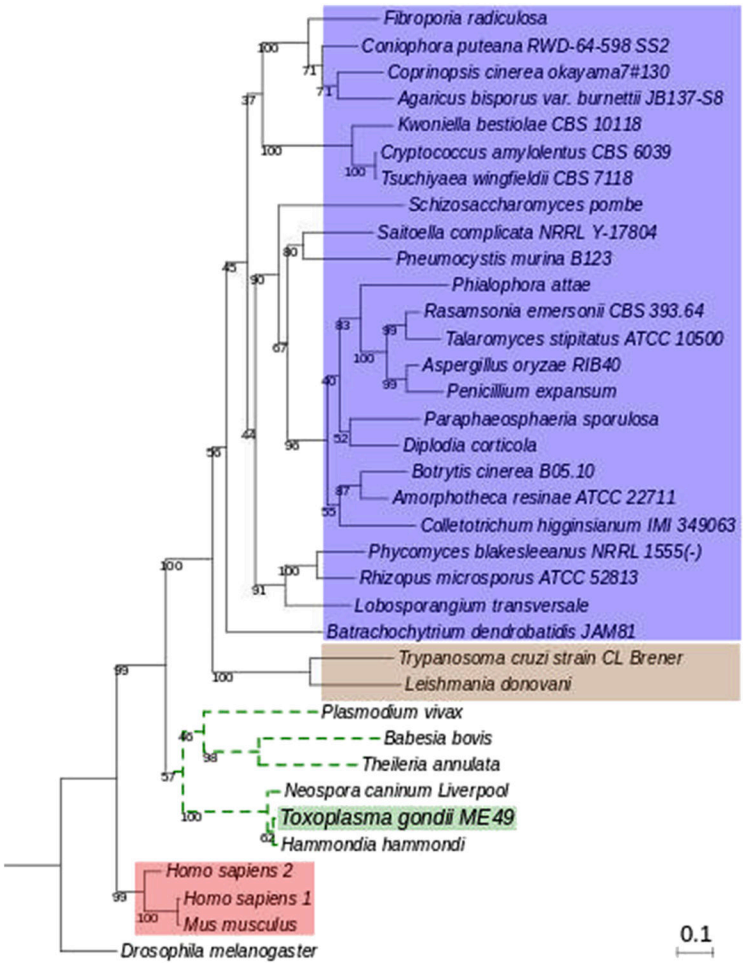

Figure 1. Phylogenetic analysis of TGME49_209160 amino acid sequence.

Phylogenetic tree representing evolutionary relationships between the TGME49_209160 amino acid sequence and reference sequences of known and predicted NMTs of eukaryotic organisms such as other apicomplexans (dotted green lines), trypanosomatids (brown box), mammals (red box) and fungi (blue box). Sequence of *Drosophila melanogaster* was included as outgroup.

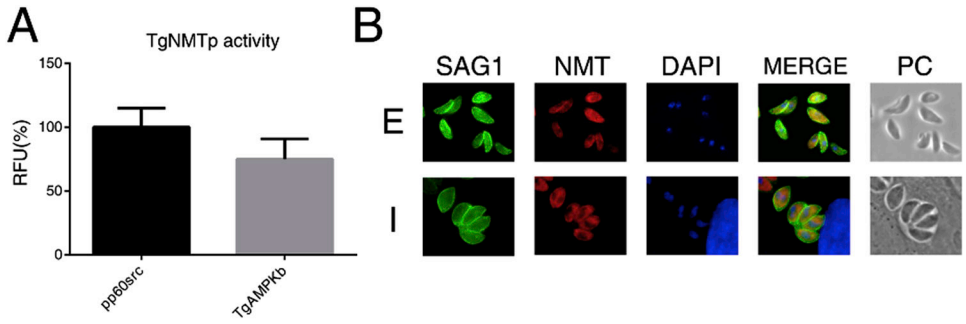

Figure 2. Enzymatic activity and cellular localization of TgNMT.

(A) TgNMTp was incubated with either with a standard substrate (pp60src) or a putative *T. gondii* substrate (TgAMPKb; access number Toxodb: TGME49_268960) as described in the Material and Methods section. Activity is expressed as a percentage of the relative fluorescence units (RFU) obtained with pp60src. Appropriate blanks (without Myristoyl-CoA or substrate) are already subtracted. **(B)** Indirect Immunofluorescence assay using the anti-TgNMT serum (NMT, red) on intra- (I) or extracellular (E) parasites. Mouse anti-SAG1 (SAG1, green) was used to label the plasma membrane and DAPI was used to mark nuclei. Parasites were observed by phase-contrast microscopy (PC).

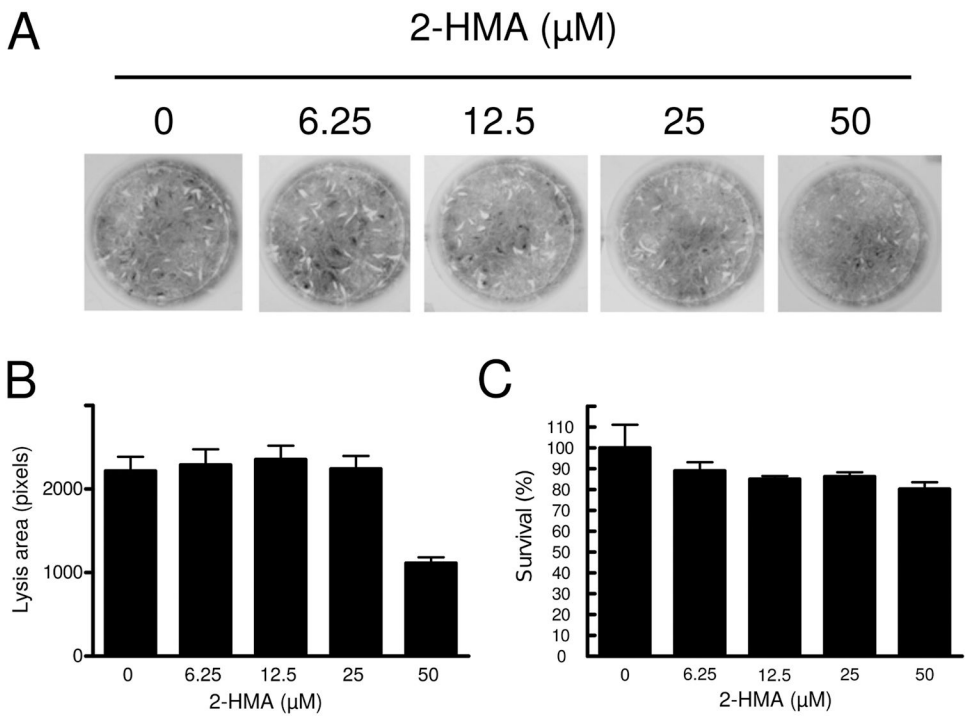

Figure 3. NMT activity is necessary for *T. gondii* lytic cycle.

(A) Plaque lysis assay performed on HFF monolayers infected with tachyzoites and incubated in presence of 0 (vehicle) or increasing concentrations of 2HMA. **(B)** Quantitation of lysis area. **(C)** Cellular cytotoxicity and of increasing concentrations of 2HMA was evaluated on HFF monolayers through the reduction of MTT. The experiments were carried out in duplicate in three independent experiments. Asterisk indicates significant differences with $p < 0.05$.

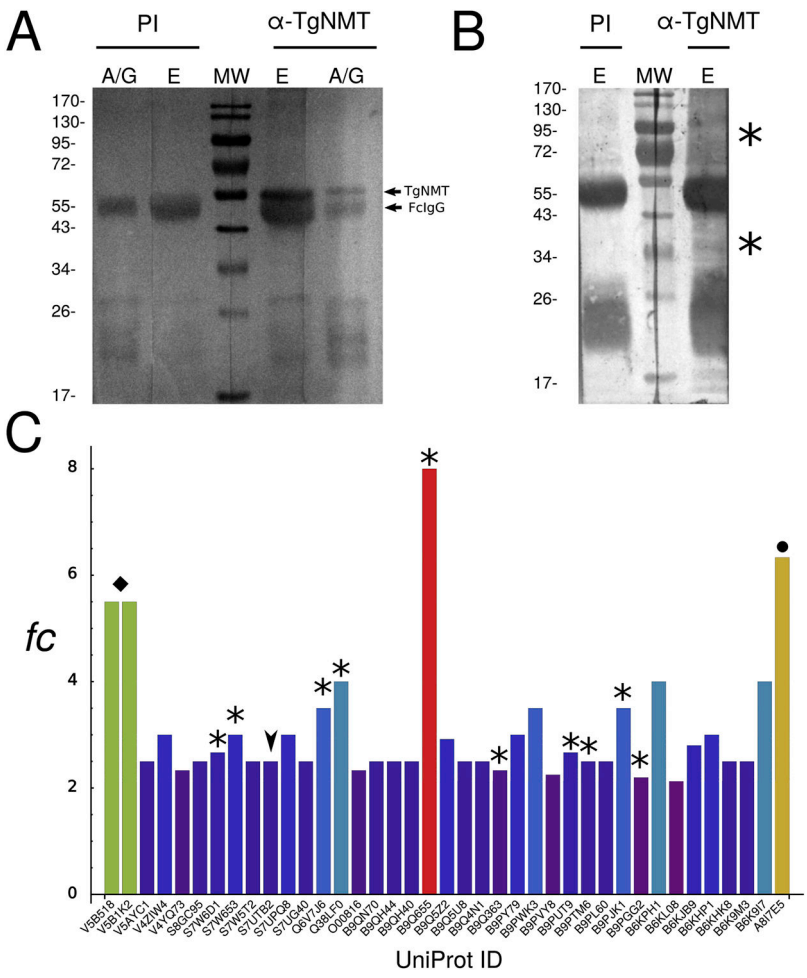

Figure 4. TgNMT interactome.

(A) Western blot analysis of *T. gondii* NMT immunoprecipitation with TgNMT antiserum (α-TgNMT). The pre-immune fraction (PI) was used as negative control. A/G: proteins bound to A/G sepharose beads even after elution; E: eluted proteins from with A/G sepharose; MW: molecular weight pattern. TgNMT migration is indicated with an arrowhead. **(B)** Ponceau red stain of Western blot described above. Asterisks indicate bands corresponding to potential TgNMT partners. **(C)** Bar chart of spectral count (SpC) for every protein detected in the MudPIT analysis. Only those proteins whose SpC fold change (fc) exceeded the value of 2 were plotted. Asterisks indicate proteins that participate in the translation process. Arrowheads indicate a predicted TgNMT substrate. A black diamond indicates proteins that participate in vesicular transport. A black circle indicates GRA7 antigen, a well-represented antigen in the total proteome.

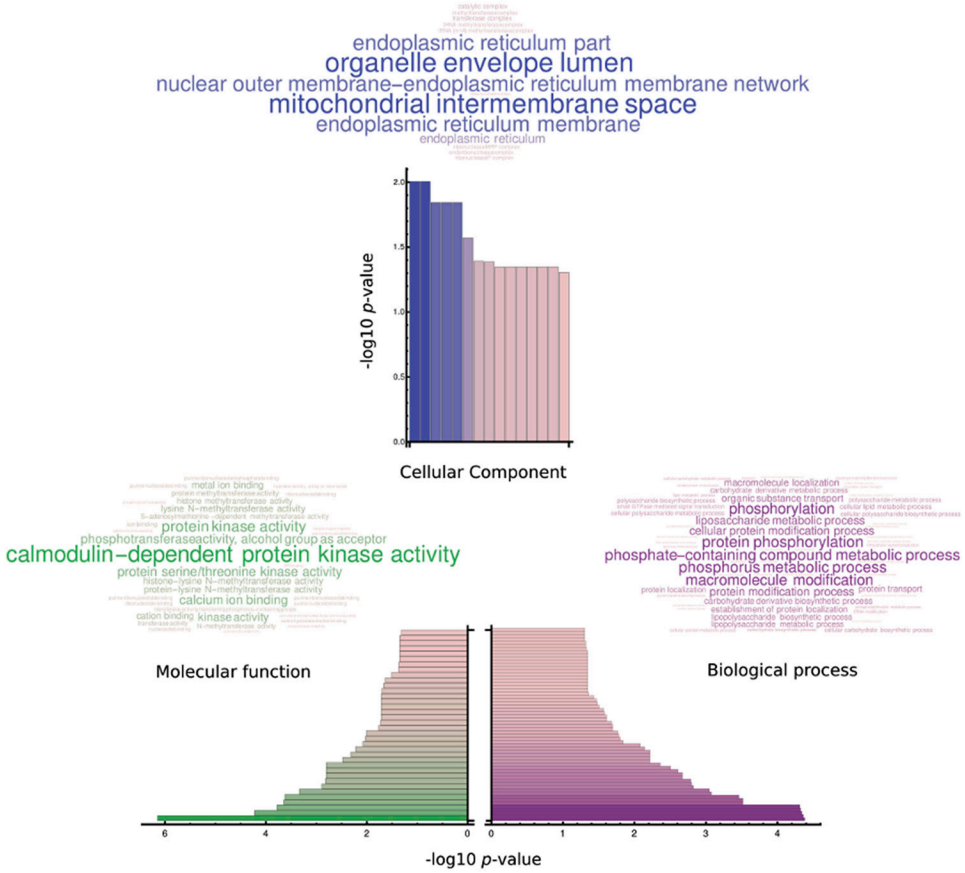

Figure 5. Gene Ontology (GO) analysis of the *in silico* myristoylome.

Possible biological process affected **(A)**; predicted molecular function involved **(B)** and putative cellular components affected **(C)** by protein myristoylation.

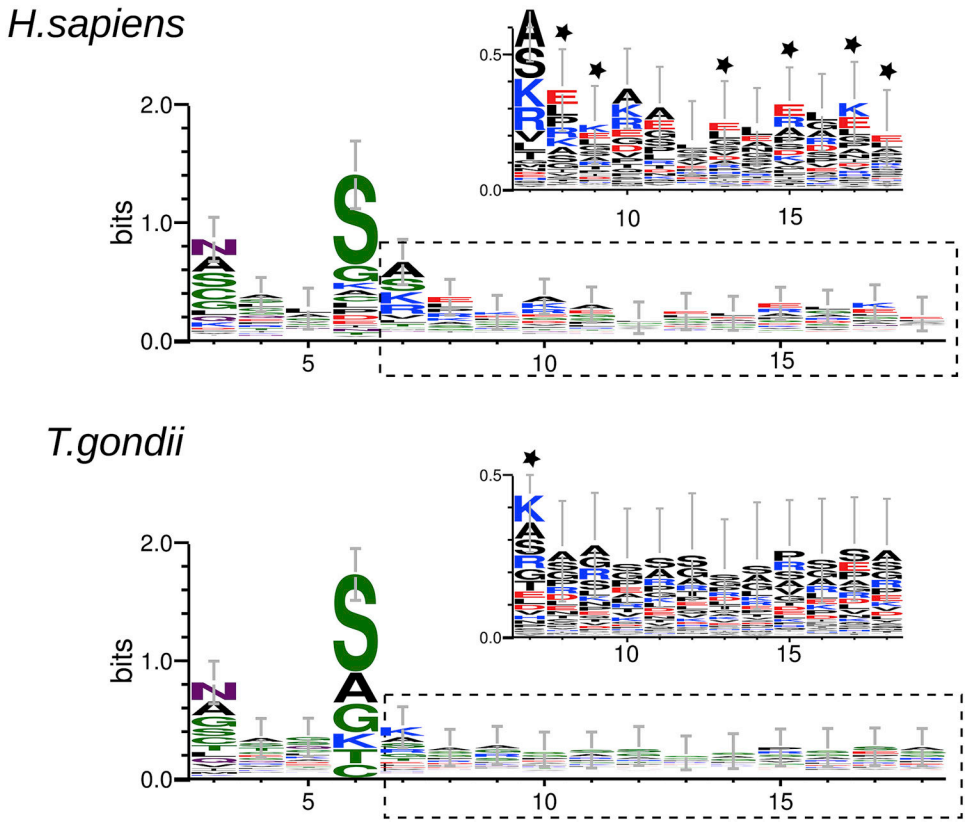

Figure 6. Web logo of HsNMT and TgNMT peptides substrates.

Amino acid alignment of HsNMT and TgNMT peptide substrates from positions 3 to 18. Residues are highlighted according to their chemistry (polar: green; neutral: purple; basic: blue; acidic: red; hydrophobic: black). For both enzymes, a zoomed view over positions 6 to 18 (dotted lines) is shown; residues are highlighted by charge (neutral: black; positive: blue; negative: red). Stars indicate differences.

Table 1.

Selected putative myristoylated proteins

Accession ID*	Description	Protein Function	Fitness score^	Report on experimental myristoylation
TGME49_207460	Rab 5b	Intracellular membrane trafficking	−1.35	(Ebine, et al., 2016) Ψ
TGME49_223940	GAP45	Gliding motility	−4.14	(Frenal, et al., 2010)
TGME49_237820	ISP2	Parasite division	−2.04	(Beck, et al., 2010)
TGME49_245710	phosphatidylinositol-4-phosphate 5-kinase, putative	Calcium signaling	1.15	(Wright, et al., 2015) #
TGME49_249970	acylated pleckstrin-homology domain-containing protein (APH)	Microneme exocytosis	−5.53	(Bullen, et al., 2016)
TGME49_260820	ISP1	Parasite division	−2.04	(Beck, et al., 2010)
TGME49_262860	ADP-ribosylation factor family protein 1, putative	Dense granule proteins release	−0.52	(Wright, et al., 2015)#
TGME49_268960	5'-AMP-activated protein kinase subunit beta-1 family protein, putative	Energy sensor	−3.95	(Wright, et al., 2016, Wright, et al., 2015) #,&
TGME49_269780	ADP-ribosylation factor, putative	Vesicle biogenesis 32	−1.28	(Wright, et al., 2015) #
TGME49_281580	PP2C-like, putative	Protein phosphatase	−1.06	(Wright, et al., 2016, Wright, et al., 2015) #,&
TGME49_305860	CDPK3	Parasite egress	−0.09	(Garrison, et al., 2012)
TGME49_316540	ISP3	Parasite division	−0.63	(Beck, et al., 2010)

*Toxodb release 40 (15 Oct 2018).

^Data represent mean CRISPR phenotype scores reported by Sidik *et al.* (Sidik, et al., 2016)

#Reported to be myristoylated in *L. donovani*

ΨReported to be myristoylated in *P. falciparum*

&Reported to be myristoylated in *T. brucei*