Published in final edited form as:

J Eukaryot Microbiol. 2018 July ; 65(5): 709–721. doi:10.1111/jeu.12626.

Tagging of Weakly Expressed *Toxoplasma gondii* Calcium-Related Genes with High-Affinity Tags

Miryam A. Hortua Triana^{a,*}, Karla M. Márquez-Nogueras^{a,*}, Le Chang^{a,1}, Andrew J. Stasic^a, Catherine Li^a, Kevin A. Spiegel^{a,2}, Amrita Sharma^{a,b}, Zhu-Hong Li^a, and Silvia N.J. Moreno^{a,b}

^aCenter for Tropical and Emerging Global Diseases, University of Georgia, Athens, Georgia, 30602, USA

^bDepartment of Cellular Biology, University of Georgia, Athens, Georgia, 30602, USA

Abstract

Calcium ions regulate a diversity of cellular functions in all eukaryotes. The cytosolic Ca^{2+} concentration is tightly regulated at the physiological cytosolic concentration of 50-100 nM. The *Toxoplasma gondii* genome predicts the presence of several genes encoding potential Ca^{2+} channels, pumps and transporters. Many of these genes are weakly expressed and likely tightly regulated due to their potential impact to the physiology of the cell. Endogenous tagging has been widely used to localize proteins in *T. gondii* but low level of expression of many of them makes visualization of tags difficult and sometimes impossible. The use of high performance tags for labeling proteins expressed at low level is ideal for investigating the localization of these gene products. We designed a Carboxy-terminus tagging plasmid containing the previously characterized “spaghetti monster-HA” (smHA) or “spaghetti monster-MYC” (smMYC) tags. These tags consist of 10 copies of a single epitope (HA or MYC) inserted into a darkened green fluorescence protein scaffold. We localized six proteins of various levels of expression. Clonal lines were isolated and validated by PCR, western blot and immunofluorescence analyses. Some gene products were only visible when tagged with smHA and in one case the smHA revealed a novel localization previously undetected.

Keywords

Toxoplasma; Calcium; spaghetti monster; C-terminus tagging; phospholipase C; Calcineurin; Hedgehog; high affinity tags

TOXOPLASMA gondii is an Apicomplexan protist that infects a wide variety of vertebrate hosts, including humans. *Toxoplasma* is recognized as a major opportunistic pathogen in

Correspondence: S. Moreno, Department of Cellular Biology and Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA 30602, USA. Telephone number: +1-542-4736; FAX: +1 542-9493; smoreno@uga.edu.

¹Current address: College of Veterinary Medicine, Jilin University, Changchun, China

²Current address: College of Veterinary Medicine, University of Georgia

*These authors contributed equally to this work

DR. SILVIA MORENO (Orcid ID : 0000-0002-2041-6295)

Hortua Triana et al.---Spaghetti Monster Tags for Localizing Toxoplasma Proteins

immunocompromised patients (Israelski and Remington 1993, Luft et al. 1984) as well as the cause of congenital (Wong and Remington 1994) and ocular (Holland 2004) toxoplasmosis in immunocompetent hosts. These parasites rely on calcium signaling for vital functions like gliding motility (Lovett and Sibley 2003), conoid extrusion (Del Carmen et al. 2009), microneme secretion (Carruthers et al. 1999, Lovett et al. 2002, Carruthers and Sibley 1999), host cell invasion (Lovett and Sibley 2003, Vieira and Moreno 2000) and host cell egress (Garrison et al. 2012, Borges-Pereira et al. 2015). However, information on the molecules, effectors, and mechanisms involved is fragmented or missing. In addition, a large number of Ca^{2+} related genes remain uncharacterized. The available information on Ca^{2+} storage and function in Apicomplexans, although fragmented, points toward the presence of unique Ca^{2+} mediated pathways (Lourido and Moreno 2015).

The genome of *T. gondii* is known (Lorenzi et al. 2016) but approximately half of the genes are annotated as hypothetical of unknown function and 30-40% of the *Toxoplasma gondii* genes may be essential (Sidik et al. 2016). The cellular localization of proteins provides information that could help in understanding their potential function and is usually tested using immunofluorescence assays with specific antibodies against the protein of interest or against a previously attached tag most often to the 3' region of the gene. In *Toxoplasma* C-terminal tagging of genes has provided an essential tool that has greatly increased the knowledge of the cellular biology of this important pathogen (Huynh and Carruthers 2009).

Protein tags are usually peptide antigens (epitopes) or fluorescent proteins like green fluorescent protein (GFP), and their main advantage is the availability of reliable commercial antibodies against the peptides or their easy detection by fluorescence microscopy in case of GFP and derivatives. Endogenous tagging is the method of choice because it shows the endogenous levels of the protein of interest and prevents artifacts due to overexpression. However, some proteins such as ion channels and transporters are weakly expressed and multiple copies of the epitope tags are sometimes required to detect them. Viswanathan et al. (Viswanathan et al. 2015) recently reported an engineered group of highly antigenic tags based on GFP-like fluorescent proteins in which numerous copies of peptide epitopes are incorporated into the N- and C-termini and internal loops of the superfolder version of GFP. The strategy developed by Viswanathan et al. (Viswanathan et al. 2015) was to use GFP as scaffold because of its capacity to accept large insertions without affecting proper folding and cell trafficking (Abedi et al. 1998, Kiss et al. 2006). In addition, the superfolder version helped to improve antigenicity and allowed simultaneous IgGs binding to different locations of the tag protein (Pedelacq et al. 2006). As a final modification they added a tripeptide GGG mutation to the chromophore to convert these GFP variants into non-fluorescent proteins (Barondeau et al. 2003), and named these tags as "spaghetti monster fluorescence proteins" (smFP) (Viswanathan et al. 2015). We incorporated smFP into the plasmid used for the Ligation Independent Cloning (pLIC) tagging system of *T. gondii* (Huynh and Carruthers 2009) and also used them with the CRISPR/Cas9 tagging system.

We present in this work the results of tagging *T. gondii* genes predicted from the genome database to be expressed at high levels and genes predicted to be weakly expressed. We

selected genes potentially important for Ca^{2+} signaling. This technique will have wide application for the localization of non-abundant proteins in *T. gondii*.

MATERIALS AND METHODS

Parasite culture

All parasite strains were continuously maintained in vitro by serial passage in Dulbecco's modified minimal essential media (DMEM) with 1% FBS, 2.5 µg/ml amphotericin B, 100 µg/ml streptomycin in the human telomerase reverse transcriptase immortalized foreskin fibroblasts (hTERT) (Farwell et al. 2000). Carboxy-terminus tagging was done in the parental line RHTati ku80 (Tati ku80) (Sheiner et al. 2011), a parasite line that contains the tetracyclin-regulated transactivator system that allows conditional expression of genes (Meissner et al. 2001) and also in which the *ku80* gene was deleted increasing efficiency of homologous recombination (Fox et al. 2009; Huynh and Carruthers 2009).

Construction of plasmids and parasite lines

The vector p3xHA.LIC.CAT used for in situ C-terminal tagging (a generous gift from B. Striepen, UGA) was modified by replacing the 3HA tag with the spaghetti monster tag smHA or smMYC (Viswanathan et al. 2015) (plasmids generously shared by L. Looger, Howard Hughes Medical Institute). The 3HA fragment was excised with NdeI and AvrII restriction enzymes and replaced with 1 kb smFP gene tag amplified with primers that contain the same restriction sites. Constructs were validated by enzymatic digestion using PacI and XhoI and sequencing. Plasmids were linearized with PacI (restriction site in LIC cassette) and the correct size was verified by gel electrophoresis.

The specific tagging plasmid was created by ligation independent cloning as previously described (Huynh and Carruthers 2009). Briefly, a homology region of approximately 1 kb covering the 3' region of the gene of interest excluding the STOP codon was amplified by PCR using *T. gondii* RH genomic DNA as template. The PCR product was cloned into the psmHA.LIC.CAT and p3HA.LIC.CAT plasmids (Huynh and Carruthers 2009, Sheiner et al. 2011). Positives clones were selected by restriction digest and confirmed by sequencing. The oligonucleotides primers used for creating the gene-tagging plasmids and for PCR validations are listed in Table S1.

Prior to transfection all plasmids were linearized within the region of homology (Huynh and Carruthers 2009). Approximately 20 µg of plasmid DNA was transfected into 1×10^7 *T. gondii* strain Tati ku80 using a Gene Pulser X Cell electroporator (BioRad, Hercules, CA, USA). Selection for integration of the targeting plasmid was performed with 20 µM chloramphenicol, and clones were isolated by limiting dilution. DNA of stable clones was isolated and screened by PCR using primers upstream of the homology region (F_{GOI} primer) and downstream into the psmHA.LIC.CAT or p3HA.LIC.CAT plasmids (R_{val} primer) (Fig. 1). The primers used for validation for every gene are shown in Table S1.

For the smMYC plasmid, a fragment corresponding to the single guide RNA (sgRNA) against the C-terminal (near the stop codon) of the *Toxoplasma* calcineurin subunit A (TgCnA) gene was cloned into the pU6-Universal plasmid (a gift from Sebastian Lourido,

MIT) (Sidik et al. 2014). The smMYC cassette (smMYC tag plus CAT resistant cassette) was amplified by PCR from the psmMYC.LIC.CAT plasmid and primers containing 50 bp homology arms of the region upstream and downstream of the stop codon of the *TgCnA* gene. This homology arms were added to promote homology recombination in the Tati ku80 strain. The resulting pU6::TgCnA plasmid was co-transfected with the smMYC cassette (3:1 respectively) into RH and Tati ku80 tachyzoites. Selection followed with chloramphenicol for 7 days. Parasites were sub-cloned by limiting dilution and screening for clones was done by PCR. Validation of the correct insertion of the tag was done by immunofluorescence and western blots.

For quantitative PCR, total RNAs from the 3HA and smHA tagged lines and Tati ku80 was extracted and reversed-transcribed into cDNA. The qPCR reaction was done using the iQTMSYBR Green master mix (BioRad, Hercules, CA, USA), plus primers and the reversed-transcribed cDNA (Primers are shown in Table S1). The qRT-PCR was carried out on a CFX96TM PCR Real-Time detection system (C1000TouchTM Thermal cycler, BioRad, Hercules, CA, USA). Relative quantification software (CFX MaestroTM software) was used for the analysis and relative expression levels were calculated as the fold change using the formula 2^{-C_T} (Livak et al. 1995). Normalization was done using Actin primers as reference. Experiments were repeated three times with triplicate samples.

Western blot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed established protocols (Laemmli 1970). Lysates were prepared by resuspending a pellet of 1×10^8 tachyzoites in 50 μ l of Cell Lytic^M lysis buffer containing 12.5 U benzonase and 1 X protease cocktail inhibitor (P8340 Sigma, St. Louis, MO, USA). The reaction was stopped with one volume of 2% SDS and 1 mM EDTA. Total lysates were boiled in Laemmli sample buffer (BioRad, Hercules, CA, USA).

Immunoblotting followed established protocols using rat anti-HA monoclonal antibody (1:1,000) or mouse anti-MYC (1:1,500) (Santa Cruz, Dallas, TX, USA). Detection was done with horseradish peroxidase-conjugated (HRP) anti-Rat IgG or anti-Mouse IgG secondary antibodies (Life Technologies-Thermo Fisher Scientific, Waltham, MA, USA) at 1:10,000. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) using the SuperSignal West Pico chemiluminescent substrate kit (Pierce-Thermo Fisher Scientific, Waltham, MA, USA). Loading control for westerns were done with primary mouse-anti-tubulin antibodies at a 1:15,000 dilution and anti-mouse IgG-HRP as secondary. For some immunoblots, the Odyssey Clx LICOR system was used for detection and the secondary antibodies were replaced by goat anti-mouse IRDye®800WC (1:10,000).

Immunofluorescence microscopy

Extracellular parasites were collected and purified as previously described (Liu et al. 2014). Parasites were washed once with buffer A with glucose (BAG, 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose and 50 mM HEPES, pH 7.4) and an aliquot with 2×10^4 parasites was overlaid on a coverslip previously treated with poly-L-Lysine. Intracellular tachyzoites were grown on hTERT cells on coverslips previously infected with freshly lysed

parasites. Both extracellular and intracellular parasites were fixed with 2.5% paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.25% Triton X-100, blocked with 3% bovine serum albumin (BSA), and exposed to primary antibodies (rat anti-HA 1:100, mouse anti-MYC 1:100). The secondary antibodies used were goat-anti-rat or goat-anti-mouse Alexa-Fluor488 (Life Technologies- Thermo Fisher Scientific, Waltham, MA. USA) at 1:1,000. For co-localization studies rabbit anti-SAG1 (provided by John Boothroyd, Stanford University) at 1:1,000 was used for plasma membrane, mouse anti- SERCA 1,1000 (from David Sibley) (Nagamune et al. 2007) and mouse anti-tubulin (Sigma T5168) (1:200) to label the cytoskeleton, and mouse anti-GFP (molecular probes A11120) to label centrin2 after transfection with the GFP-TgCentrin2 fusion plasmid (provided by Ke Hu, University of Indiana)(Hu et al. 2006). Secondary antibodies were goat anti-mouse or goat anti-rabbit Alexa-Fluor 596, respectively. Slides were examined using an Olympus IX-71 inverted fluorescence microscope with a photometric CoolSNAP HQ charge-coupled device (CCD) camera driven by DeltaVision software (Applied Precision, Seattle, WA). Super resolution images were collected with the Elyra S1 super resolution structured illumination microscopy system (Zeiss, Oberkochen, Germany).

Immunoprecipitation

Freshly lysed tachyzoites expressing the tagged gene TgGT1_247370-3HA and smHA were collected and filtered through an 8 µm membrane (Whatman). Tachyzoites were washed twice in BAG and resuspended in a lysis buffer (20mM HEPES, pH 7.4, 150 mM NaCl, 0.5% NP40 and 1% Triton X-100 with two tablets of EDTA-free protease inhibitor cocktail) to a final concentration of 2×10^9 total cells. Lysis was allowed to proceed for thirty minutes at 4 °C and cells were centrifuged at $21,000 \times g$ for 30 minutes.

Immunoprecipitation of the 3HA and smHA tagged TgGT1_247370 protein was performed using the Pierce HA Tag/Co-IP kit (Thermo Fisher Scientific, Waltham, MA. USA) according to the manufacturer's instructions. Briefly, HA magnetic beads were washed twice in lysis buffer and mixed with the parasite lysate by vortexing for 1 hour at RT. Beads were collected and the flow-through fraction was saved for further analysis. Beads were washed twice in lysis buffer and once in ddH₂O by gentle mixing. The supernatant fraction was saved. TgGT1_247370 tagged protein was recovered with the elution buffer from the Pierce HA Tag/Co-IP kit and mixing for 10 minutes at RT. Supernatant was collected and the pH neutralized with neutralization buffer. The final sample was used for electrophoresis gel and western blot analysis.

RESULTS

Localization of *Toxoplasma* genes with high-performance tags

We categorized genes into high and low expression using the RNA sequencing data from ToxoDB (Gajria et al. 2008) according to the transcript level of fragments per kilobase of exon model per million mapped reads (FPKM). Genes with transcript expression levels under 40 FPKM were considered to be expressed at low level (Table 1). The reference used was the Tachyzoite Transcriptome Time Series (*T. gondii* GT1 Tachyzoite mRNA Illumina sequence aligned to the ME49 genome) (Lorenzi et al. 2016). Ranking for the expression of

the genes is also presented under percentile (%) available from ToxoDB. Considering this transcript expression for each gene and the experimental observation from our studies we developed an expression scale presented in Table 1 (Expression column). Evidence of expression from the mass spectrometry data was also taken into consideration. We selected six genes for the validation of the spaghetti monster strategy, which are shown in Table 1. Three of these genes had been already studied and three are annotated as hypothetical proteins but predicted to function in signaling as channels or receptors.

We modified the ligation independent cloning (LIC) plasmid p3HA.LIC.CAT (Huynh and Carruthers 2009) to build a C-terminal tagging plasmid with the “spaghetti monster” fluorescent protein smHA or smMYC. A fragment between the NdeI and AvrII restriction sites corresponding to the 3HA tag was removed and replaced with the smHA gene tag (1 kb) (Fig. 1A). Linearized plasmids containing the smHA and 3HA showed a size of ~6 kb and ~5 kb respectively (Fig. 1B). The strategy used for gene tagging via a single homology recombination event is shown in Fig. 1C. The homology region (HR) of the GOI was fused to the tag gene. Validation of this insertion at the genomic level in the selected clones was done using specific primers (F_{GOI} and R_{pVAL}) as well as sequencing of the integration site.

We tagged two genes expressed at high level: TgGT1_311310 (calcineurin subunit A, serine/threonine phosphatase 2B catalytic subunit, TgCnA), and TgGT1_305050 (calmodulin putative- essential light chain, ELC2). Calcineurin is a Ca²⁺-calmodulin dependent protein phosphatase that plays an important role in calcium signaling (Crabtree 2001). In *Toxoplasma* and *Plasmodium*, calcineurin has been shown to be involved in host cell engagement and attachment (Paul et al. 2015, Philip and Waters 2015). TgELC2 is a calcium-binding protein that forms part of the glideosome complex and interacts with MyoA, a type XIV myosin. The glideosome complex is essential for parasite motility and for host cell invasion and egress (Williams et al. 2015).

We used ligation independent cloning (LIC) to construct a TgCnA plasmid for C-terminal tagging with a 3HA epitope and with smHA. We transfected the linearized plasmids into Tati ku80 tachyzoites and after selection and subcloning we isolated clonal lines. Validation of correct tag insertion was done by PCR (Fig. 2A) and expression of the tagged protein was confirmed by western blot analysis with commercial anti-HA antibodies. Total lysates of clonal parasites expressing the TgCnA-smHA gene showed a band at 96 kDa (predicted size ~56 kDa + ~39 kDa of the smHA) while lysates of parasites expressing the TgCnA-3HA gene showed a band at 59 kDa (Fig. 2C). Immunofluorescence analysis (IFA) using fluorescence (Fig. 2B) or super-resolution microscopy (Fig. 2D) showed cytosolic localization of TgCnA-3HA and TgCnA-smHA in both extracellular and intracellular tachyzoites (Fig. 2B). This localization was in agreement with previous results using live tachyzoites expressing TgCnA tagged with m-CherryRFP (Paul et al. 2015) and showed that the smHA tag did not alter the endogenous localization of the TgCnA. Plaque assays showed that the TgCnA-smHA tagged parasites had a modest defect in growth (30-40% smaller plaques) when compared to the parental line (Fig. S1A-B). Growth of the TgCnA-3HA did not show a significant difference with growth of the parental line (Fig. S1A-B). *TgCnA* is an essential gene (Paul et al, 2015) and the growth difference could be because the larger tag could interfere with proper trafficking. The expression level of TgCnA was not affected as

evaluated by qRT-PCR with primers targeting the TgCnA gene and compared with the expression of TgCnA from the Tati ku80 line (Fig. S1C). Controls for the specific amplification of the TgCnA-smHA is shown in Fig S1D using a combination of primers complementary to the TgCnA gene (forward primer) and the smHA tag (reverse primer). Only the cDNA isolated from the TgCnA-smHA tagged parasites showed a positive signal.

We also tagged the calmodulin-like gene TgELC2 (TgGT1_305050) and showed correct insertion of the tag into the endogenous locus by PCR (Fig. 2E). The expression was confirmed by IFA with anti-HA (Fig. 2F) and by western blot analysis with anti-HA antibodies, which showed bands of 60 and 20 kDa for the smHA and 3HA-tagged protein, respectively (Fig. 2G). The IFAs showed plasma membrane localization of TgELC2 in both extracellular and intracellular tachyzoites (Fig. 2F & 2H). Previous work using 3HA-ELC2 tagged parasites showed peripheral co-localization with GAP45 (Williams et al. 2015). Our results showed that the smHA tag did not alter the endogenous localization of TgELC2 even considering that the size of the tag is as large as the protein. TgCnA and TgELC2 are highly expressed genes and we only observed sharper fluorescence with the TgCnA-smHA and TgELC2-smHA cells. The images with smHA and 3HA tagged were acquired under identical microscope settings but we did not perform fluorescence quantification. Viswanathan et al, showed that spaghetti monster tags possess higher antigenicity using fluorescence correlation spectroscopy and showed increased brightness (Viswanathan et al. 2015). These cells grew as the wild type parasites as evaluated by plaque assays (Fig. S1A, B). The expression of the gene was not affected by the presence of the sm tag as evaluated by qPCR (Fig. S1E).

Localization of proteins expressed at low level

We tested the smHA tag in two hypothetical genes (TgGT1_222060 and TgGT1_290870) for which the RNAseq data available in the genome database (ToxoDB) predicts low level of expression (Table 1). TgGT1_222060 is annotated as encoding a hypothetical protein, nevertheless its gene sequence contained charged regions analogous to voltage sensor domains present in calcium voltage-gated channels, making this gene a candidate calcium channel (Prole and Taylor 2011). The TgGT1_222060 gene sequence predicted to encode for a protein of 1162 amino acids with six transmembrane domains and no signal peptide (Fig. 3A). The predicted topology based on its amino acid sequence using the TMHMM server 2.0 and Protter for visualization is shown in Fig. 3A. Domain predictions were described according to the InterProScan method used for ToxoDB.

Following a similar procedure to that described above we generated clonal cell lines of parasites expressing TgGT1_222060 tagged at the C-terminus with either the 3HA or the smHA. Insertion in the correct endogenous locus of the 3HA or the smHA tag was confirmed by PCR (Fig. 3B). Bands size of 1.8 kbp and 2.8 kbp were obtained, respectively. Western blot analysis of lysates obtained from tachyzoites expressing TgGT1_222060-smHA showed a band that appear to run closer to the ~140 kDa mark which is approximately 30 kDa lower than the expected size (~165 kDa). The predicted size for TgGT1_222060 is 127 kDa plus the smHA molecular weight (~38.8 kDa). This small discrepancy could be the result of the lack of accuracy of the protein markers used or N-

terminus processing of the protein or simply the annotation may not be accurate. The database prediction for the same gene in the ME49 strain (TgME49_222060) is 103 kDa, which agrees with our western data. More experiments would be necessary to solve this discrepancy. No signal was detected from lysates of tachyzoites expressing the 3HA-tagged TgGT1_222060 (Fig. 3C). This was likely because of the low level of expression of this gene. The expression level of the smHA gene was not altered by the presence of the tag as shown by qRT-PCR (Fig. S2A). IFAs revealed a vesicular localization of the smHA tagged protein while no evident labeling was observed from either intracellular or extracellular tachyzoites expressing the 3HA-tagged gene (Fig. 3D).

TgGT1_290870 was predicted to be similar to the protein patched homolog 1 (PTCH1), which is a member of the Patched family. These proteins are associated with the Hedgehog signaling pathway, an important signaling pathway that is involved in transmission of information for proliferation (Lee et al. 2016). The patched protein interact with Smoothened, a G-protein coupled receptor (GPCR), which transduces the activation signal to downstream pathway components (Arensdorf et al. 2016). An interplay between hedgehog and calcium and inositol phosphate signaling was shown in the developing spinal cord (Belgacem and Borodinsky 2011). It remains to be elucidated, which is the role of these genes homologues of regulators of animal development in *Toxoplasma*. The amino acid sequence of TgGT1_290870 predicts twelve transmembrane domains with four of them predicted to be part of a sorting sensing domain (SSD) (Fig. 3E, *gray*). Another domain (indicated in pink in the cartoon in Fig. 3E) is predicted to have similarity to the RND family of transporters, which are bacterial multidrug resistance transporters (Fig. 3E). The TM domains that are not part of the patched family domain are shown in green. The predicted topology based on its amino acid sequence was done using TMHMM server 2.0 and Protter for visualization.

Following a similar procedure to that described above we generated clonal cell lines of parasites expressing TgGT1_290870 tagged at their C-terminus with either the 3HA or the smHA. We confirmed the correct insertion of the 3HA and smHA tag by PCR (Fig. 3F). Western blot analysis of total lysates showed a band around ~170 kDa, but no band was observed in western blots of lysates from tachyzoites expressing the 3HA tagged TgGT1_290870 (Fig. 3G). The expression level of the tagged gene with the smHA tag was not significantly different from the 3HA tagged gene (Fig. S2D). IFA of the smHA tagged gene revealed plasma membrane localization but very weak signals were observed in cells expressing the 3HA tagged TgGT1_290870 (Fig. 3H).

In summary, in both cases (TgGT1_222060 and TgGT1_290870) PCR analysis confirmed the insertion in the correct locus. Western blot and immunofluorescence analyses failed to show any evidence of expression of the 3HA tagged version of these genes. However, the smHA-tagged TgGT1_222060 and TgGT1_290870 proteins showed vesicular and plasma membrane localization, respectively, and western blot analysis showed signals of the correct predicted sizes.

We also studied a gene predicted to be a transient receptor potential channel (TRP) (TgGT1_247370). TRP channels generally conduct cations and they possess six

transmembrane domains (TM) (Venkatachalam and Montell 2007). In mammals cells TRP channels have been shown to participate in Ca^{2+} entry and in release of intracellular stores when they are located in organelles (Gees et al. 2010). The *T. gondii* genome predicts for the presence of two genes with TRP-like features (Prole and Taylor 2011) and both are predicted to be expressed at very low levels. The TgGT1_247370 gene is predicted to generate a protein of 1261 amino acid (~140 kDa) with 13 TM domains (Fig. 4A). We tagged the TgGT1_247370 gene with 3HA and smHA tags and first validated correct integration of the tag by PCR (Fig. 4B). Western blot analysis with anti-HA antibodies revealed a band of ~130 kDa in lysates of the parasites expressing the TgGT1_247370-smHA gene while no signal was observed in lysates of parasites expressing the TgGT1_247370-3HA gene (Fig. 4C). The predicted size for the TgGT1_247370 is 140 kDa plus the smHA tag. The signal observed in our Westerns appeared to correspond to a smaller protein. This discrepancy could be due to 327 extra base-pairs found in the sequence of TgGT1_247370 (Fig. S3). We investigated the source of this extra sequence and we found that part is from an intron located between exons 1 and 2. This fragment would result in a different open reading frame where the start codon could be further downstream of the predicted start codon, resulting in a smaller protein of probably less than 130 kDa. The difference observed in size could be due to both the fact that the gene is likely miss-annotated and also to post-translational processing.

With the aim to evaluate if the presence of the large tag would affect growth of the parasites, we performed plaque assays for the three lines, TgGT1_222060-smHA, TgGT1_290870-smHA and TgGT1_247370-smHA and compared them with the corresponding 3HA of each line and with the parental strains (Fig. S2, B,D,F). We observed no difference in plaque sizes between the different lines.

We next tested the possibility of using the smHA-tagged proteins for affinity purification. We immunoprecipitated the tagged TRP-like protein using HA beads and run the eluted sample in an SDS gel (Fig. 4D). The parental strain was also used as control for the immunoprecipitation and was run in a parallel gel. A band of ~130 was observed in the TRP smHA elute that was neither observed in the 3HA-tagged line or the parental cell lysate. IFAs of parasites expressing the TgGT1_247370-smHA gene showed vesicular localization around the nucleus of extracellular tachyzoites (Fig. 4E). No signal was observed in IFAs of parasites expressing the TgGT1_247370-3HA gene (not shown). We also performed co-localization IFAs with the Endoplasmic Reticulum marker TgSERCA (Nagamune et al. 2007) to further investigate the localization of TgGT1_247370 (Fig. 4E) and we found that the HA signal co-localized with the ER. It would be interesting to determine if this TRP-like channel localized to the ER is involved in release or leakage of Ca^{2+} to the cytosol of *Toxoplasma*.

A novel localization for the phosphoinositide phospholipase C disclosed by high-performance tags

A phosphoinositide specific phospholipase C, which catalyzes the conversion of phosphatidyl inositol bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol (DAG), has been characterized in *T. gondii* (TgPI-PLC, TgGT1_248830)

(Fang et al. 2006, Bullen et al. 2016). The protein was found to localize to the plasma membrane of both intracellular and extracellular tachyzoites using polyclonal anti-TgPI-PLC antibodies. However, western blot analysis of tachyzoite lysates showed a very weak signal with these antibodies (Fang et al. 2006). We transfected Tati ku80 cells with the pLIC plasmid prepared for C-terminal tagging with 3HA and smHA. We were able to obtain transfectants expressing the TgPI-PLC-smHA while the TgPI-PLC-3HA did not survive the selection. We confirmed the insertion of the tag into the TgPI-PLC genomic locus by PCR (Fig. 5A). Western blot analysis of lysates of tachyzoites expressing the smHA tagged protein showed a band of ~160 kDa, which agrees with the predicted size of the protein plus the smHA tag (Fig. 5B). IFAs showed some peripheral localization (Fig. 5C), as previously reported (Fang et al. 2006, Bullen et al. 2016). Interestingly, an additional localization was evident in the parasites expressing the smHA tag. Six dots at the apical end of both intracellular and extracellular parasites (Fig. 5C) were clearly evident, which was confirmed by super-resolution microscopy (Fig. 5D). We compared this result with IFAs of tachyzoites expressing 3Ty-tagged TgPI-PLC (kindly provided by Dominique Soldati, Université de Genève) with anti-Ty antibody, and the six dots at the apical region were observed although weakly stained and easily confused with random labeling of vesicles (Fig. 5E). We further characterized the TgPI-PLC localization using markers for the cytoskeleton (tubulin), which showed no co-localization with TgPI-PLC (Fig. 5F). However, an almost perfect co-localization was observed with *T. gondii* centrin 2 (TgCentrin2). TgCentrin2 is an EF-hand containing calcium-binding protein previously reported to localize to the centrioles and to form a peripheral annuli of six dots in the pre-conoidal ring in mature tachyzoites (Hu et al. 2006).

In conclusion, smHA-tagging revealed a novel localization for TgPI-PLC (Fig. 5F), which was not evident in previous studies. The role of a PI-PLC at this location is intriguing and deserves further investigation.

Use of high performance MYC tags with the CRISPR/Cas9 approach

C-terminal tagging using CRISPR/Cas9 has been used in *T. gondii* (Sidik et al. 2014, Shen et al. 2017). We examined the feasibility of tagging genes with the spaghetti monster tag using this genome editing strategy. A scheme of this approach using the calcineurin gene TgCnA (TgGT1_311310) is shown in Fig. S4. Co-transfection of pU6::TgCnA and the smMyc plasmids was successful in both Tati ku80 and RH strains. Twelve out of twelve smMyc-tagged clones tested positive by PCR in Tati Ku80, and nine out of twelve clones of the smMyc tagged RH strain were positive. However, from these 9 RH positive clones only two were positive by western blot analysis (data not shown). TgCnA-smMyc tagged positive clones were validated first by PCR showing a band at ~770 bp (Fig. 6A) and expression was confirmed by western blot analysis (Fig. 6B). Anti-Myc antibodies recognized a band size of ~110 kDa similar to the band size observed when TgCnA was tagged with smHA (Fig. 2C). Immunofluorescence analysis using extracellular and intracellular parasites showed identical cytosolic localization (Fig. 6C) as observed previously with smHA tagged tachyzoites (Fig. 2B and D).

DISCUSSION

Our work demonstrates that spaghetti monster tagging in *T. gondii* tachyzoites can be used for the localization of proteins expressed at low levels. We constructed two spaghetti monster C-terminal tagging plasmids, one with 10 copies of the HA epitope and another one with 10 copies of the MYC epitope. The tags were cloned from the original spaghetti monster tags designed by the group of Loren Looger (Viswanathan et al. 2015) and inserted into the *Toxoplasma* LIC plasmids designed for C-terminal tagging (Huynh and Carruthers 2009) (Sheiner et al. 2011). We first tested smHA for the localization of known proteins like calcineurin 2B catalytic subunit (TgCnA) (TgGT1_311310), which localizes to the cytoplasm (Paul et al. 2015) and the essential light chain protein (ELC2) (TgGT1_305050), which localize to the plasma membrane (Williams et al. 2015). In both of these cases the smHA tagged genes produced proteins of the correct size and localization.

The strategy significantly improved the localization of proteins expressed at low levels for which no expression evidence could be obtained with conventional HA tags. This was the case for three hypothetical genes TgGT1_222060, TgGT1_290870 and TgGT1_247370. The 3HA tagged versions of these genes did not show evidence of expression by either western blot or immunofluorescence analyses. However, the smHA-tagged proteins showed vesicular, peripheral, plasma membrane or endoplasmic reticulum localization, respectively. We also tested the smHA tagged TgGT1_247370 in an affinity purification assay and for this we first immunoprecipitated the tagged protein with anti-HA antibodies and run western blots for validation. This strategy could work for enrichment of some proteins expressed at very low levels. We did not test this high affinity tags in co-immunoprecipitation assays but we suspect that they could potentially increase affinity and provide cleaner results in the identification of interacting proteins. There are some limitations to the use of these tags and for some genes expressed at very low levels we were unable to confirm their expression and localization. This was the case for the EF hand domain containing protein TgGT1_223520 with an FPKM predicted to be below 5 (ToxoDB) (Fig. S5). It could be that the gene is not expressed in tachyzoites, which is the form that we used in these studies. The other limitation is that, because of its size, the tag (~40 kDa) could interfere with normal trafficking for those proteins that have targeting signals at their C terminus. We tested two genes for which the HA also showed evidence of expression and for these cases the tagged proteins had the same localization as the HA-tagged ones indicating that for these cases the large smHA tag did not affect localization.

Spaghetti monster tags revealed a novel and unexpected localization for the phosphoinositide phospholipase C. This enzyme generates the second messenger inositol 1,4,5-trisphosphate (InsP₃), which in mammalian cells gates the endoplasmic reticulum InsP₃ receptor, which opens and allows Ca²⁺ to flow into the cytosol (Berridge and Irvine 1984). The *Toxoplasma* enzyme (TgPIPLC, TgGT1_248830) was reported to localize to the plasma membrane despite the absence of transmembrane domains (Fang et al. 2006). Probably the enzyme translocates to the plasma membrane, where it meets its substrate phosphatidylinositol bisphosphate (PIP₂) and this results in the activation of Ca²⁺ signaling. We detected an additional localization of TgPIPLC to six dots at the apical region of the parasites where it co-localizes with centrin2 (Hu et al. 2006). This novel localization could

be important and reveal a novel role for this enzyme in cell division. Very little information is available for the role of PI-PLCs in cell division and this aspect deserves further investigation (Cauvin and Echard 2015).

In conclusion, the modular nature of smHA scaffolds and the availability of several derivatives containing other antigenic epitopes like FLAG and V5 (Viswanathan et al. 2015), will greatly expand our ability to localize *T. gondii* proteins with low levels of expression. This approach can potentially be expanded for the localization of weakly expressed proteins in other protozoan parasites like *Trypanosoma cruzi* as we report in the accompanying communication (Cruz-Bustos et al. 2018).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sarada Viswanathan and Loren Looger for the Spaguetti monster plasmids, Dominique Soldati for the *Toxoplasma i pipic-myc* mutants, Ke Hu for the Centrin2-GFP plasmid, David Sibley for the anti-SERCA antibody and John Boothroyd for the SAG antibody. Beejan Asady and Melissa Storey provided technical assistance. We thank Dr. Muthugapatti K. Kandasamy and the Biomedical Microscopy Core (BMC), Coverdell, UGA for the use of the microscopes and also for training and advise. This work was supported by U.S. National Institutes of Health grants AI1096836 and AI128356 to SNJM. KMN was supported in part by a fellowship from the T32 training grant to the Center for Tropical and Emerging Global Diseases (AI060546) and LC was supported by The China Scholarship Council (CSC) as a Visiting scholar for two years at University of Georgia. KAS was partially supported by a summer training grant from the NIH National Center for Research Resource Grant Number 05T35OD010433.

LITERATURE CITED

- Abedi MR, Caponigro G, Kamb A. Green fluorescent protein as a scaffold for intracellular presentation of peptides. *Nucleic Acids Research*. 1998; 26:623–630. [PubMed: 9421525]
- Arendsdorf AM, Marada S, Ogden SK. Smoothed Regulation: A Tale of Two Signals. *Trends Pharmacol Sci*. 2016; 37:62–72. [PubMed: 26432668]
- Barondeau DP, Putnam CD, Kassmann CJ, Tainer JA, Getzoff ED. Mechanism and energetics of green fluorescent protein chromophore synthesis revealed by trapped intermediate structures. *Proc Natl Acad Sci U S A*. 2003; 100:12111–6. [PubMed: 14523232]
- Belgacem YH, Borodinsky LN. Sonic hedgehog signaling is decoded by calcium spike activity in the developing spinal cord. *Proc Natl Acad Sci U S A*. 2011; 108:4482–7. [PubMed: 21368195]
- Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*. 1984; 312:315–21. [PubMed: 6095092]
- Borges-Pereira L, Budu A, McKnight CA, Moore CA, Vella SA, Hortua Triana MA, Liu J, Garcia CR, Pace DA, Moreno SN. Calcium Signaling throughout the *Toxoplasma gondii* Lytic Cycle: A STUDY USING GENETICALLY ENCODED CALCIUM INDICATORS. *J Biol Chem*. 2015; 290:26914–26. [PubMed: 26374900]
- Bullen HE, Jia Y, Yamaryo-Botte Y, Bisio H, Zhang O, Jemelin NK, Marq JB, Carruthers V, Botte CY, Soldati-Favre D. Phosphatidic Acid-Mediated Signaling Regulates Microneme Secretion in *Toxoplasma*. *Cell Host Microbe*. 2016; 19:349–60. [PubMed: 26962945]
- Carruthers VB, Sibley LD. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol*. 1999; 31:421–8. [PubMed: 10027960]
- Carruthers VB, Moreno SN, Sibley LD. Ethanol and acetaldehyde elevate intracellular [Ca²⁺] and stimulate microneme discharge in *Toxoplasma gondii*. *Biochem J*. 1999; 342(Pt 2):379–86. [PubMed: 10455025]

- Cauvin C, Echard A. Phosphoinositides: Lipids with informative heads and mastermind functions in cell division. *Biochim Biophys Acta*. 2015; 1851:832–43. [PubMed: 25449648]
- Crabtree GR. Calcium, calcineurin, and the control of transcription. *J Biol Chem*. 2001; 276:2313–6. [PubMed: 11096121]
- Cruz-Bustos T, Moreno SNJ, Docampo R. Detection of Weakly Expressed *Trypanosoma cruzi* Membrane Proteins Using High-Performance Probes. *J Eukaryot Microbiol*. 2018
- Del Carmen MG, Mondragon M, Gonzalez S, Mondragon R. Induction and regulation of conoid extrusion in *Toxoplasma gondii*. *Cell Microbiol*. 2009; 11:967–82. [PubMed: 19416276]
- Fang J, Marchesini N, Moreno SN. A *Toxoplasma gondii* phosphoinositide phospholipase C (TgPI-PLC) with high affinity for phosphatidylinositol. *Biochem J*. 2006; 394:417–25. [PubMed: 16288600]
- Farwell DG, Shera KA, Koop JI, Bonnet GA, Matthews CP, Reuther GW, Coltrera MD, McDougall JK, Klingelhutz AJ. Genetic and epigenetic changes in human epithelial cells immortalized by telomerase. *Am J Pathol*. 2000; 156:1537–47. [PubMed: 10793065]
- Fox BA, Ristuccia JG, Gigley JP, Bzik DJ. Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end joining. *Eukaryot Cell*. 2009; 8:520–9. [PubMed: 19218423]
- Gajria B, Bahl A, Brestelli J, Dommer J, Fischer S, Gao X, Heiges M, Iodice J, Kissinger JC, Mackey AJ, Pinney DF, Roos DS, Stoeckert CJ Jr, Wang H, Brunk BP. ToxoDB: an integrated *Toxoplasma gondii* database resource. *Nucleic Acids Res*. 2008; 36:D553–6. [PubMed: 18003657]
- Garrison E, Treeck M, Ehret E, Butz H, Garbuz T, Oswald BP, Settles M, Boothroyd J, Arrizabalaga G. A Forward Genetic Screen Reveals that Calcium-dependent Protein Kinase 3 Regulates Egress in *Toxoplasma*. *PLoS Pathogens*. 2012; 8:e1003049. [PubMed: 23209419]
- Gees M, Colasoul B, Nilius B. The role of transient receptor potential cation channels in Ca²⁺ signaling. *Cold Spring Harb Perspect Biol*. 2010; 2:a003962. [PubMed: 20861159]
- Holland GN. Ocular toxoplasmosis: a global reassessment. *American Journal of Ophthalmology*. 2004; 137:1–17. [PubMed: 14700638]
- Hu K, Johnson J, Florens L, Fraunholz M, Suravajjala S, DiLullo C, Yates J, Roos DS, Murray JM. Cytoskeletal Components of an Invasion Machine—The Apical Complex of *Toxoplasma gondii*. *PLOS Pathogens*. 2006; 2:e13. [PubMed: 16518471]
- Huynh MH, Carruthers VB. Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryot Cell*. 2009; 8:530–9. [PubMed: 19218426]
- Israelski DM, Remington JS. Toxoplasmosis in Patients with Cancer. *Clinical Infectious Diseases*. 1993; 17:S423–S435. [PubMed: 8274608]
- Kiss C, Fisher H, Pesavento E, Dai M, Valero R, Ovecka M, Nolan R, Phipps ML, Velappan N, Chasteen L, Martinez JS, Waldo GS, Pavlik P, Bradbury ARM. Antibody binding loop insertions as diversity elements. *Nucleic Acids Research*. 2006; 34:e132–e132. [PubMed: 17023486]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–5. [PubMed: 5432063]
- Lee RT, Zhao Z, Ingham PW. Hedgehog signalling. *Development*. 2016; 143:367–72. [PubMed: 26839340]
- Liu J, Pace D, Dou Z, King TP, Guidot D, Li ZH, Carruthers VB, Moreno SN. A vacuolar-H(+) -pyrophosphatase (TgVP1) is required for microneme secretion, host cell invasion, and extracellular survival of *Toxoplasma gondii*. *Mol Microbiol*. 2014; 93:698–712. [PubMed: 24975633]
- Livak KJ, Marmaro J, Todd JA. Towards fully automated genome-wide polymorphism screening. *Nat Genet*. 1995; 9:341–2. [PubMed: 7795635]
- Lorenzi H, Khan A, Behnke MS, Namasivayam S, Swapna LS, Hadjithomas M, Karamycheva S, Pinney D, Brunk BP, Ajioka JW, Ajzenberg D, Boothroyd JC, Boyle JP, Darde ML, Diaz-Miranda MA, Dubey JP, Fritz HM, Gennari SM, Gregory BD, Kim K, Saeij JP, Su C, White MW, Zhu XQ, Howe DK, Rosenthal BM, Grigg ME, Parkinson J, Liu L, Kissinger JC, Roos DS, Sibley LD. Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic *Toxoplasma gondii* genomes. *Nat Commun*. 2016; 7:10147. [PubMed: 26738725]
- Lourido S, Moreno SN. The calcium signaling toolkit of the Apicomplexan parasites *Toxoplasma gondii* and *Plasmodium* spp. *Cell Calcium*. 2015; 57:186–93. [PubMed: 25605521]

- Lovett JL, Sibley LD. Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells. *J Cell Sci.* 2003; 116:3009–16. [PubMed: 12783987]
- Lovett JL, Marchesini N, Moreno SN, Sibley LD. *Toxoplasma gondii* microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores. *J Biol Chem.* 2002; 277:25870–6. [PubMed: 12011085]
- Luft BJ, Brooks RG, Conley FK, McCabe RE, Remington JS. Toxoplasmic encephalitis in patients with acquired immune deficiency syndrome. *JAMA.* 1984; 252:913–917. [PubMed: 6748191]
- Meissner M, Brecht S, Bujard H, Soldati D. Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. *Nucleic Acids Res.* 2001; 29:E115. [PubMed: 11713335]
- Nagamune K, Beatty WL, Sibley LD. Artemisinin induces calcium-dependent protein secretion in the protozoan parasite *Toxoplasma gondii*. *Eukaryot Cell.* 2007; 6:2147–56. [PubMed: 17766463]
- Paul AS, Saha S, Engelberg K, Jiang RH, Coleman BI, Kosber AL, Chen CT, Ganter M, Espy N, Gilberger TW, Gubbels MJ, Duraisingh MT. Parasite Calcineurin Regulates Host Cell Recognition and Attachment by Apicomplexans. *Cell Host Microbe.* 2015; 18:49–60. [PubMed: 26118996]
- Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and characterization of a superfolder green fluorescent protein. *Nat Biotech.* 2006; 24:79–88.
- Philip N, Waters Andrew P. Conditional Degradation of Plasmodium Calcineurin Reveals Functions in Parasite Colonization of both Host and Vector. *Cell Host & Microbe.* 2015; 18:122–131. [PubMed: 26118994]
- Prole DL, Taylor CW. Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites. *PLoS One.* 2011; 6:e26218. [PubMed: 22022573]
- Sheiner L, Demerly JL, Poulsen N, Beatty WL, Lucas O, Behnke MS, White MW, Striepen B. A systematic screen to discover and analyze apicoplast proteins identifies a conserved and essential protein import factor. *PLoS Pathog.* 2011; 7:e1002392. [PubMed: 22144892]
- Shen B, Brown K, Long S, Sibley LD. Development of CRISPR/Cas9 for Efficient Genome Editing in *Toxoplasma gondii*. *Methods Mol Biol.* 2017; 1498:79–103. [PubMed: 27709570]
- Sidik SM, Hackett CG, Tran F, Westwood NJ, Lourido S. Efficient genome engineering of *Toxoplasma gondii* using CRISPR/Cas9. *PLoS One.* 2014; 9:e100450. [PubMed: 24971596]
- Sidik SM, Huet D, Ganesan SM, Huynh MH, Wang T, Nasamu AS, Thiru P, Saeij JP, Carruthers VB, Niles JC, Lourido S. A Genome-wide CRISPR Screen in *Toxoplasma* Identifies Essential Apicomplexan Genes. *Cell.* 2016; 166:1423–1435 e12. [PubMed: 27594426]
- Venkatachalam K, Montell C. TRP channels. *Annu Rev Biochem.* 2007; 76:387–417. [PubMed: 17579562]
- Vieira MCF, Moreno SNJ. Mobilization of intracellular calcium upon attachment of *Toxoplasma gondii* tachyzoites to human fibroblasts is required for invasion. *Molecular and Biochemical Parasitology.* 2000; 106:157–162. [PubMed: 10743619]
- Viswanathan S, Williams ME, Bloss EB, Stasevich TJ, Speer CM, Nern A, Pfeiffer BD, Hooks BM, Li WP, English BP, Tian T, Henry GL, Macklin JJ, Patel R, Gerfen CR, Zhuang X, Wang Y, Rubin GM, Looger LL. High-performance probes for light and electron microscopy. *Nat Methods.* 2015; 12:568–76. [PubMed: 25915120]
- Williams MJ, Alonso H, Enciso M, Egarter S, Sheiner L, Meissner M, Striepen B, Smith BJ, Tonkin CJ. Two Essential Light Chains Regulate the MyoA Lever Arm To Promote *Toxoplasma* Gliding Motility. *MBio.* 2015; 6:e00845–15. [PubMed: 26374117]
- Wong SY, Remington JS. Toxoplasmosis in pregnancy. *Clin Infect Dis.* 1994; 18:853–61. quiz 862. [PubMed: 8086543]

Figure 1. Spaghetti monster high affinity tag. **A.** Strategy used for the smHA plasmid construction. A fragment between the NdeI and AvrII restriction sites corresponding to the 3HA tag in the p3HA.LIC.CAT plasmid was removed and replaced with the smHA gene tag. **B.** Linearized plasmids containing the smHA and 3HA tags showed a size of ~5.9 kb and ~4.9 kb, respectively (red arrows). **(C)** Scheme showing the strategy used for gene tagging via a single homology recombination event. F_{GOI} and R_{pVAL} indicate the position of the primers used for validation of tag insertion for each gene locus. Primers are shown in Table S1. HR,

homology region, TUB, tubulin promoter, GOI, gene of interest, CAT, chloramphenicol acetyl transferase, resistance conferring gene U, uncut, C, cut.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2.

The spaghetti monster tag does not alter protein localization. **A.** PCR analysis showing the correct insertion of the 3HA or smHA tag at the 3' end of the TgCnA-3HA (3HA) (1.6 kbp) and TgCnA-smHA (smHA) (2.6 kbp) parasites. The PCR primers used are shown in Table S1. **B.** Cytosolic localization of TgCnA as observed with αHA antibodies (green). Extracellular and intracellular tachyzoites expressing TgCnA-HA and TgCnA-smHA are shown. The nucleus was stained with DAPI (blue). Scale bars = 5 μm. **C.** Western blot of lysates obtained from tachyzoites expressing TgCnA-3HA and TgCnA-smHA. Anti-HA

antibodies detected bands of ~59 (3HA) or ~96 (smHA) kDa. The parental (P) line Tati ku80 was used as negative control. α -tubulin antibody was used for loading control. **D.** Super-resolution microscopy shows cytosolic localization of TgCnA in both tagged lines. **E.** PCR analysis showing the correct insertion of the 3HA (3HA) (1.5 kbp) or smHA (smHA) (2.5 kbp) tags at the 3' region of the *TgELC2* gene. PCR products were absent in DNA samples obtained from WT tachyzoites. The PCR primers used are shown in Table S1. **F.** Immunofluorescence microscopy using α HA antibodies (green) confirmed plasma membrane localization in both extracellular and intracellular parasites expressing both tagged versions of TgELC2. Scale bars = 5 μ m. **G.** Western blot analysis using α HA antibodies detected bands of the expected size (~18 (3HA) and ~55 (smHA) kDa). Tubulin was used as loading control. **H.** Super-resolution microscopy images of TgELC2-3HA (3HA) and TgELC2-smHA (smHA) expressing cell lines, showing plasma membrane localization as detected by is-HA antibodies (green). Scale bars = 5 μ m.

Figure 3.

High affinity tags improved detection of proteins expressed at low levels. **A.** Predicted topology of TgGT1_222060 protein based on its predicted amino acid sequence using the TMHMM server 2.0 and Protter for visualization. Transmembrane domains predicted to be part of a voltage-gated channel are shown in purple. **B.** PCR analysis showing the correct insertion of the 3HA (3HA) (1.8 kbp) or smHA (smHA) (2.8 kbp) tags at the 3' region of the *TgGT1_222060* gene. The PCR primers used are shown in Table S1. **C.** Western blot analysis of TgGT1_222060-3HA (3HA) and TgGT1_222060-smHA (smHA) expressing

cell lines. Anti-HA antibodies detected a band of ~140 kDa in smHA tachyzoite lysates (black arrow). No band was detected in lysates from 3HA cells. Lysates of the Tati ku80 parental line were used as negative controls. α -tubulin was used as loading control. **D.** Immunofluorescence microscopy using α -HA antibodies (green) show cytosolic vesicular localization in extracellular and intracellular parasites expressing TgGT1_222060-smHA but no signals in parasites expressing TgGT1_222060-3HA. Nuclei are stained with DAPI (blue). Scale bars are 5 μ m. **E.** Predicted Topology of the TgGT1_290870 protein from its predicted amino acid sequence using the TMHMM server 2.0 and Protter. Domains of the full patch family were colored in yellow and TM domains part of the SSD are gray while the TM domains part of RNA family are shown in pink. **F.** PCR analysis showing the correct insertion of the 3HA (3HA) (1.9 kbp) or smHA (smHA) (2.9 kbp) tags at the 3' region of the *TgGT1_29080* gene. The PCR primers used are shown in Table S1. **G.** Western blot analysis using α -HA antibodies detected a band of the expected size (~170 kDa) in lysates of the smHA-tagged tachyzoites but no band was observed in lysates of the 3HA-tagged cells. Tubulin was used as loading control. **H.** Immunofluorescence microscopy using α -HA antibodies (green) detected plasma membrane localization in both extracellular and intracellular parasites expressing TgGT1_290870-smHA. Very weak signals were observed in the 3HA-tagged strain. Nuclei were stained with DAPI (blue). Scale bars = 5 μ m.

Figure 4.

Spaghetti monster high affinity tag can be used for immunoprecipitation assays. **A.** Predicted topology of the TgGT1_247370 protein amino acid sequence using the TMHMM server 2.0 and Protter. The PKD_channel Polycystin cation domain is shown in orange. **B.** PCR analysis showing the correct insertion of the 3HA (3HA) (1.6 kbp) or smHA (smHA) (2.6 kbp) tags at the 3' region of the *TgGT1_247370* gene. The PCR primers used are shown in Table S1. **C.** Western blot analysis using α-HA antibodies detected a band of ~130 kDa in smHA-tagged tachyzoites (black arrowhead) and no band in 3HA-tagged cells. Tubulin was used as loading control. **D.** Western blot analysis of the immunoprecipitation using α-HA antibodies showed a band of the same size in the elution sample (E) as indicated by the black arrowhead. No band was observed in the flow-through (FT) and elution (E) of the 3HA-tagged cells. The parental line Tati ku80 was used as a negative control and no band was detected. **E.** IFAs with α-HA antibodies (green) shows punctate staining around the ER that co-localizes with the reaction of α-SERCA (ER marker) (red) in extracellular parasites expressing TgGT1_247370-smHA. Parasite nuclei were labeled with DAPI (blue). Scale bars = 5 μm.

Figure 5.

Spaghetti monster tag reveals a novel localization for TgPI-PLC. **A.** PCR analysis showing the correct insertion of the 3HA or smHA tags at the 3' region of the TgPI-PLC gene. A DNA fragment of 1.5 kbp was amplified. **B.** Western blot analysis of lysates obtained from parasites expressing TgPI-PLC-smHA. Anti-HA antibodies detected a band of ~160 kDa (SM). The parental (P) line Tati ku80 was used as negative control. The α -tubulin antibody was used as loading control. **C.** IFA with α -HA antibodies of extracellular and intracellular tachyzoites expressing TgPI-PLC-smHA showing peripheral localization and an additional dotted localization at the apical end (arrow). **D.** Super-resolution microscopy image reveal six dots at the apical end using α -HA antibodies (green) in smHA tagged parasites. Nuclei are labeled with DAPI (blue). **E.** IFA using α -Ty antibodies (green) to stain 3Ty tagged parasites. A weak signal at the apical end is observed (white arrow). DAPI (blue) labels the nuclei. **F.** Super resolution microscopy images of smHA-tagged parasites double labeled with α -HA antibodies (green) and α -GFP antibodies (red) showing co-localization with centrin2 (top) but not with cytoskeleton marker tubulin (red). Scale bars on C, D, E and F = 5 μ m.

Figure 6.

CRISPR-Cas9 dependent C-terminal endogenous tagging with the high affinity tag smMyc. **A.** PCR analysis showing the correct insertion of the smMyc (smMyc) tag at the 3' region of the *TgCnA* gene. A DNA fragment of 770 bp was amplified from smMyc-tagged tachyzoites. **B.** Western blot analysis of TgCnA-smMyc expressing cell lines. Anti-Myc antibodies detected a band of ~110 kDa (smMyc). The parental (P) line Tati ku80 was used as negative control. α-tubulin antibody was used as loading control. **C.** IFA using anti-Myc antibodies (green) to label the cytosol of smMyc-tagged extracellular and intracellular parasites. DAPI (blue) labels the nuclei. Scale bars = 5 μm.

Table 1

Genes tagged in this study and comparison of expression levels

GENE ID	NAME	FPKM	PERCENTILE (%) ⁵	EXPRESSION ⁶	TRANSMEMBRANE DOMAINS	PREDICTED SIZE (KDA)	LOCALIZATION
TgME49_311310	Calcineurin 2B Catalytic subunit (TgCnA) ¹	30-60	~ 75	++++	0	56.4	Cytosol
TgME49_305050	Essential light chain 2 (ELC2) ²	100-400	~ 87.5	+++++	0	15.2	Plasma membrane
TgME49_222060	Hypothetical protein	15-25	~ 50	+++	6	127.8	Vesicular
TgME49_290870	Patched protein family	15-40	~ 62.5	+++	12	130.5	Plasma membrane
TgME49_247370	Hypothetical protein	2.4-20	~ 25	+	13	140.9	Endoplasmic reticulum
TgME49_248830	Phosphoinositide phospholipase C ³	8-15	~ 37.5	++	0	122	Plasma membrane/Centrin-colocalization
TgME49_209030	Actin (ACT1) ⁴	600-800	~ 87.5	+++++	0	41.9	-
TgME49_200320	Hypoxanthine xanthine guanine phosphoribosyl transferase (HXGPRT) ⁴	100-200	~87.5	+++++	0	31.4	-
TgME49_312480	Uracil phosphoribosyl transferase ⁴	30-60	~ 75	++++	0	27.6	-

¹Paul et al., 2015

²William et al., 2015

³Fang et al., 2015

⁴Genes not studied in this paper only used as reference of expression

⁵Ranking of expression of genes compare to all other in the Transcriptome experiment (ToxoDB)

⁶Scale of expression