Published in final edited form as:

J Eukaryot Microbiol. 2018 July ; 65(5): 722–728. doi:10.1111/jeu.12517.

Detection of Weakly Expressed *Trypanosoma cruzi* Membrane Proteins Using High-Performance Probes

Teresa Cruz-Bustos^a, Silvia N.J. Moreno^{a,b}, and Roberto Docampo^{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

Membrane proteins in trypanosomatids are, in general, weakly expressed and confirmation of their subcellular localization frequently requires their overexpression with epitope tags. However, overexpression can lead to mislocalization of the probes. Viswanathan et al. (*Nat. Methods*, 2015) described high performance tags for localization of weakly expressed proteins. We report here the use of these protein tags, named “spaghetti monster”, for CRISPR /Cas9-mediated C-terminal endogenous tagging of *Trypanosoma cruzi* to localize two weakly expressed transient receptor potential (TRP) channels to acidic compartments. The results indicate that this method will improve the detection of membrane proteins in *T. cruzi*.

Keywords

Gene tagging; acidic compartments; lysosomes; spaghetti monster tag; *Trypanosoma cruzi*

TRYPANOSOMATIDS are responsible for important diseases of humans and animals. Human infection with *Trypanosoma cruzi*, the etiologic agent of Chagas disease, is the leading cause of heart failure in Latin America. As membrane proteins are nearly 50% of current drug targets (Butt et al. 2017) their functional analysis in trypanosomes could provide important information that can be used for the development of drugs, vaccines, or diagnostic tools.

Studies on the localization of membrane proteins are important to elucidate their function. However, integral membrane proteins, which are those permanently associated to the lipid bilayer and that act as carriers or channels, are expressed in low quantities, especially when they are located in intracellular compartments. Previous studies in trypanosomes have used either antibodies or gene tagging methods (Docampo 2011). Overexpression has been used for proteins that are weakly expressed. However, overexpression could lead to mislocalization of the proteins (Lander et al. 2016b, Cruz-Bustos 2018a). Endogenous

Correspondence: R. Docampo, Department of Cellular Biology and Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA 30602, USA. Telephone number: +1-542-8104; FAX: +1 542-9493; rdocampo@uga.edu.

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

PROF. ROBERTO DOCAMPO (Orcid ID : 0000-0003-4229-8784)

tagging of *T. cruzi* has been possible only recently with the development of CRISPR/Cas9 techniques (Lander et al. 2016b, Lander et al. 2016a, Soares Medeiros et al. 2017). These endogenous tagging methods are the methods of choice in the absence of specific antibodies but their use is limited by the low expression of some proteins. In this regard, while investigating the localization of intracellular Ca^{2+} channels in trypanosomes we found that endogenous tagging of members of the transient receptor potential (TRP) superfamily of channels were almost undetectable using small epitope tags in *T. cruzi*.

The TRP superfamily of channels shares several properties like the presence of six transmembrane domains (TMDs), varying degree of sequence homology, and permeability to cations (Venkatachalam and Montell 2007). Two TRP channels have been identified in trypanosomatids with homology to the intracellular TRP mucolipin/TRP polycystin (TRPML/TRPP) mammalian channels (Prole and Taylor 2011). A gene product (TbMLP, Tb427.07.950), which has 29% identity and 49% similarity to the mouse TRPML-1 and the human TRPML-3, respectively, has been shown to be essential for optimal growth of *T. brucei* bloodstream forms (Taylor et al. 2013). The protein localizes to the lysosomes and is apparently important for iron transport, although evidence for this activity is only based on the higher susceptibility of the mutant cells to iron chelators and reactive oxygen species (ROS)-generating drugs (Taylor et al. 2013).

Viswanathan et al. (Viswanathan et al. 2015) developed a number of high performance probes consisting of green fluorescent protein scaffolds containing numerous copies of peptide epitopes that simultaneously bind IgG antibodies at each location and named them “spaghetti monsters”. This approach has several advantages over the use of multiple small epitope tags as they combine the solubility and cell tolerance of GFP-derived proteins with multiple epitopes strategically inserted in the GFP molecule thus preventing aggregation of the epitopes. In addition, the affinity of antibodies for small tags can be low and multimeric tags are frequently insufficient for detection when the protein of interest is weakly expressed (Viswanathan et al. 2015). Here we adapted the spaghetti monster tagging method using the darkened chromophore to endogenously tag ion channels and localize these low expressing channels in *T. cruzi* (TcTRP1 and TcTRP2). The results suggest that this method could have wider application in trypanosomatids.

MATERIALS AND METHODS

Chemicals and Reagents

Rabbit polyclonal antibody against serine carboxypeptidase (SCP) (Parussini et al. 2003) was a gift from Dr. Juan J. Cazzulo (University of San Martin, Argentina), the pMOTag4H vector (Oberholzer et al. 2006) was a gift from Dr. Thomas Seebeck (University of Bern, Bern, Switzerland). Q5® Hot Start High-Fidelity DNA polymerase, MagicMark™ XP Western protein standard, 1 KB Plus DNA ladder, LysoTracker Red DND-99, and Alexa-conjugated secondary antibodies were purchased from Invitrogen (ThermoFisher Scientific, Waltham, MA). Rat HA antibody was from Roche Diagnostics GmbH (Roche Applied Science, Mannheim Germany). The primers were purchased from Integrated DNA Technologies (Coralville, IO). cOmplete™, EDTA-free protease inhibitor cocktail and all other reagents of analytical grade were from Sigma (Sigma-Aldrich, St. Louis, MO).

Cell Cultures

T. cruzi Y strain epimastigotes were cultured in liver infusion tryptose (LIT) medium (Bone and Steinert 1956) containing 10% heat-inactivated fetal bovine serum at 28°C. The endogenously tagged cell lines were maintained in medium containing 250 µg/ml G418 and 200 µg/ml of hygromycin. Cells were counted using a Neubauer Chamber.

Sequence analyses

The sequence of amino acids was studied bioinformatically using the sequence obtained from TriTryp.DB. The TcTRP1 (TcCLB.503463.20) and TcTRP2 (TcCLB.510861.94) sequences were aligned using the Clustal Omega tool (Sievers et al. 2011, Goujon et al. 2010). For the prediction of domains, signal peptides and important sites we used InterPro (Finn et al. 2017) and the InterPro Scan tool (Jones et al. 2014). We also used the Phobius server (Kall et al. 2004) for transmembrane domain and signal peptide predictions. For the study of the mucolipin family in other organisms we used the Pfam (Finn et al. 2016) and UniProt databases.

Construction of plasmids

The pMOTag4H vector designed for endogenous C-terminal tagging of *T. brucei* (Oberholzer et al. 2006) was used as backbone to construct the transfection plasmids. This vector contains a 3xHA tag and the hygromycin resistance marker. The vector was modified by replacing the 3xHA tag with the spaghetti monster tag smHA (plasmid generously shared by Loren I. Looger (Janelia Research Campus, Howard Hughes Medical Institute, Ashburn, VA) (Viswanathan et al. 2015) in vector psmHA.LIC.CAT (Hortua-Triana 2018) to generate the pMOTag-SM-4H. PCR was performed with a primer set containing the SalI site (primers 1 to 5, Table S1) to amplify the *SM* from psmHA.LIC.CAT plasmid as template. PCR was done in a reaction volume of 100 µl using Q5® Hot Start High-Fidelity DNA polymerase with ~100 ng of gDNA as follows: initial denaturation for 2 min at 95°C followed by 35 cycles of 30 s at 95°C, 60 s at 60°C, 15 s at 72°C and then a final extension for 2 min at 72°C. The amplified fragment was cloned into pMOTag4H, digested using SalI enzyme and subsequently the linear plasmid was dephosphorylated to prevent re-ligation. Insert correct orientation and sequences were determined by PCR and sequencing.

Endogenous C-terminal tagging of *TcTRP1* and *TcTRP2* by CRISPR/Cas9

To obtain the C-terminal tagging of *T. cruzi* we used the Cas9/pTREX-n vector we developed (Lander et al. 2015) to clone a specific single guide RNA (sgRNA) sequence targeting the 3' end the gene. We co-transfected the 3' end-sgRNA/Cas9/pTREX-n construct with the specific DNA donor cassette amplified from the pMOTag-4H and pMOTag-SM-4H vectors to induce homology directed repair and insert a specific tag sequence (3xHA and SM) at the 3' end of the genes (TcCLB.503463.20 and TcCLB.510861.94) as described previously (Lander et al. 2016b) using primers 6 to 17 (Table S1).

Cell transfection

T. cruzi epimastigotes were grown to a density of $1-2 \times 10^7$ cells/ml, washed once with cold PBS, pH 7.4, and resuspended in ice-cold Cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM

K₂HPO₄, 2 mM EDTA, 5 mM MgCl₂, 25 mM Hepes, pH 7.6) containing 25 µg of each DNA construct in 4 mm electroporation cuvettes with 3 pulses (1.5 kV, 50 µF) delivered by a Gene Pulser Xcell (Bio-Rad). Transfectants were cultured in 5 ml LIT medium supplemented with 20% FBS and after 24 hours, the appropriate antibiotic (250 µg/ml G418 and 200 µ/ml hygromycin) was added. Stable cell lines were established and maintained under drug selection with appropriate antibiotics.

SDS-PAGE and western blot analyses

Parasites were washed twice with phosphate buffer saline (PBS) and resuspended in radio-immunoprecipitation assay buffer (RIPA: 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1% SDS, 0.1% Triton X-100) plus a tablet of cOmplete™, EDTA-free protease inhibitor cocktail. Then cells were incubated for 1 h on ice. Cell lysis was verified under a light microscope, and protein concentration was determined by Pierce BCA protein assay. Electrophoresis was performed as described previously (Cruz-Bustos 2018b). Electrophoresed proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans-blot apparatus for 1 hour at 100 V in cold conditions. Following transfer, the membrane blots were blocked with 5% non-fat dry milk in PBS containing 0.1% V/V Tween-20 (PBS-T) at room temperature during 1 hour. Blots were probed with anti-HA rat monoclonal antibody (1:1,000 dilution) and anti α -tubulin mouse antibody (1:40,000) for 1 h. After washing with PBS-T, the blots were incubated with anti-rat IgG antibody (1:20,000) or goat anti-mouse antibody (1:20,000). The membranes were washed three times with PBS-T, and western blot images were processed and analyzed using X-ray film.

Immunofluorescence analyses

The acidotrophic fluorescent dye LysoTracker Red DND-99 was used to visualize the presence of acidic compartments in fixed cells. Epimastigotes were washed once with PBS at room temperature. The cells were incubated for 30 min at 28°C in PBS containing 0.5 µM of LysoTracker Red in a dark environment. After this time, the procedure was terminated by fixing cells with 4% paraformaldehyde for 30 min. Epimastigotes without LysoTracker were washed once with PBS at room temperature and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were adhered to poly-L-lysine-coated coverslips and permeabilized for 3 min with 0.3% Triton X-100. Permeabilized cells were blocked with PBS containing 3% BSA, 1% fish gelatin, 50 mM NH₄Cl, and 5% goat serum 1 hour at room temperature. Then cells were incubated with the primary antibody (rat anti-HA tag, 1:100 and/or rabbit anti-serine-carboxypeptidase, 1:750) diluted in PBS (pH 7.4) for 1 h at room temperature. The cells were washed three times with PBS (pH 7.4) and then incubated for 1 h at room temperature in the dark with Alexa Fluor 488-conjugated goat anti-rabbit and/or Alexa Fluor 546-conjugated goat anti-rat antibody (both at 1:1,000). Following incubation with the secondary antibody, the cells were washed five times in PBS and once in water and then mounted on slides. DAPI (5 µg/ml) was included in the Fluoromount-G mounting medium to stain DNA. Controls were performed as described above but using the wild-type cell line. Differential interference contrast and fluorescence optical images were captured with a 100x objective (1.35 aperture) under nonsaturating conditions with an Olympus IX-71 inverted fluorescence microscope with a Photometrix CoolSnapHQ charge-coupled device camera driven by DeltaVision software (Applied Precision, Issaquah, WA).

and deconvolved for 15 cycles using a deconvolution software. Pixel intensity density was measured using an image processing software (FiJi, ImageJ).

Statistical analyses

All values are expressed as means $\pm$ SE. Significant differences between treatments were compared using unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$, and *n* refers to the number of experiments performed. All statistical analyses were conducted using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

RESULTS

A *T. cruzi* ortholog (TcTRP1; TcCLB.503463.20, non-Esmeraldo-like, and TcCLB.508215.6, Esmeraldo-like) to the *T. brucei* MLP channel (Taylor et al. 2013) is annotated in TriTryp.DB as putative polycystin cation channel. TcCLB.508215.6 and TcCLB.503463.20 protein sequences differ in only 7 amino acids. The other *T. cruzi* TRP (TcTRP2; TcCLB.510861.94, Esmeraldo-like, and TcCLB.503735.30, non-Esmeraldo-like) is annotated as hypothetical protein although it also has conserved predicted polycystin (PKD) domains and long TMD1-TMD2 linkers that are characteristics of TRPML and TRPP channels (Prole and Taylor 2011). The protein also has amino acids conserved in the mucolipin family. In addition, this protein has a cluster of basic residues preceding their TMD1 regions. These basic clusters are similar to the cluster responsible for binding phosphatidylinositol 3,5 bisphosphate (PI(3,5)P₂) in mouse TRPML1 (Dong et al. 2010). (Fig. S1 shows a ClustalW alignment of both protein sequences).

We used the CRISPR/Cas9-mediated endogenous C-terminal tagging of *T. cruzi* epimastigotes (Lander et al. 2016b) to introduce the 3xHA or the smHA tag sequence into the two TRP genes: TcTRP1 and TcTRP2

Fig. 1A shows that TcTRP1-3xHA and TcTRP1-SM transfectants were efficiently tagged in the endogenous locus. The corresponding bands (529, and 1557 bp, respectively) amplified with a reverse primer annealing on the hygromycin marker (Fig. 1B), are present in the resistant parasites (lanes HA and SM, respectively) but absent in the WT cells. To verify the incorporation of the tag we also used external primers (Fig. S2A). When analyzed by western blotting using commercial antibodies against HA, two bands, of ~58 and ~85 kDa were clearly detected in the TcTRP1-SM lysates (Fig. 1C). A very weak band of ~58 kDa was detected in the 3xHA-tagged cell lysates, but no bands were observed in WT cell lysates. The smaller band of ~58 kDa in the smHA-tagged cell lysates could correspond to a proteolysis product of the ~85 kDa smHA-tagged protein during lysate preparation, as the protease inhibitors used would not inhibit the hydrolysis of the glycines linking the HAs to the GFP scaffold. Immunofluorescence analysis (IFA) of TcTRP1-3xHA cells shows only weak labeling that partially co-localizes with antibodies against serine carboxypeptidase (SCP), a reservosome and lysosome marker in *T. cruzi* (Parussini et al. 2003, Sant'Anna et al. 2008). In contrast IFA of TcTRP1-SM cells shows strong reaction and very clear co-localization with SCP in all cells examined (Fig. 1D and 1E).

Using a similar approach, we found that the TcTRP2 was efficiently tagged at the endogenous locus, as detected by PCR (Fig. 1F and 1G). We also verified the incorporation of the tag using external primers (Fig. S2B). Western blot analysis (Fig. 1H) shows a band of ~99 kDa, which could correspond to partial hydrolysis of the GFP scaffold in the smHA-tagged cell lysates leaving a partially tagged TcTRP2 (as occurs with TcTRP1, Fig. 1C), and three bands of 130, 140, and 150 kDa in the TcTRP2-SM cell lysates, that could represent processing of the smHA-tagged protein when it reaches the lysosomes or post translational modifications. The strongest band is also detected in the TcTRP2-3xHA cell lysates, which also show an ~50 kDa proteolysis product. IFA of the cells shows weak labeling in all TcTRP2-3xHA transfectants that partially co-localizes with SCP (Fig. 1I and 1J). A better co-localization was observed with Lysotracker, an acidic compartment marker (Fig. S3).

DISCUSSION

Our work indicates that the high-performance probes developed by Viswanathan et al (Viswanathan et al. 2015) can be used for C-terminal tagging of genes weakly expressed in *T. cruzi*. In this work, we used a smHA probe with 10 copies of the HA epitope but the availability of other probes containing other epitopes like c-Myc, V5, and FLAG (Viswanathan et al. 2015) will be extremely useful for studies of protein-protein interaction between subunits of low expressing channel proteins.

Mutations in the mucolipin-1 channel in humans (TRPML1 or MCOLN1) are responsible for the lysosomal storage disease mucopolidosis IV, which is characterized by severe neurodegeneration. The localization of TRP channels of the mucolipin/polycystin family to acidic compartments (reservosomes and lysosomes) in *T. cruzi* suggests a role for these channels in the regulation of the ionic composition of these compartments. Previous studies using gold-labeled transferrin showed that bound transferrin initially accumulates in the cytosome of epimastigotes, traffic to the reservosomes, a prelysosomal compartment with heterogeneous content and multivesicular appearance, and then to the lysosomes (Scott 1997). Both reservosomes and lysosomes are acidic and contain SCP (Parussini et al. 2003). Our results suggest localization of TcTRP1 in both reservosomes and lysosomes (together with SCP) while TcTRP2 appears to localize only to lysosomes. Mucolipin channels act as non-selective channels permeable to various cations such as Ca^{2+} , Na^{+} and K^{+} (Di Paola et al. 2018). Vertebrate TRPML1 is also able to mobilize heavy metals such as Fe^{2+} and Zn^{2+} from the lumen of the lysosome (Dong et al. 2008, Eichelsdoerfer et al. 2010). In this regard, an ortholog of TcTRP1 has been studied in *T. brucei* (TbMLP, mucolipin channel) and proposed to be important for iron export from the lysosomes (Taylor et al. 2013). In agreement with our studies, C-terminal tagging with 12 copies of a c-Myc epitope was necessary to detect the lysosomal localization of this low expressing channel (Taylor et al. 2013).

Interestingly, TcTRP1 lacks the basic cluster of amino acids that is present in the mammalian TRPML1 channel and that is important for stimulation by $\text{PI}(3,5)\text{P}_2$, a low abundant *endo*-lysosomal specific phosphoinositide (Dong et al. 2010). However, TcTRP2 has this cluster of basic amino acids (Prole and Taylor 2011) (Fig. S1). Given the property of TRP channels to form hetero-oligomers (Di Paola et al. 2018) and the co-localization of both

TcTRP1 and TcTRP2 to lysosomes, it is tempting to speculate that they could be forming hetero-oligomers. Further work will be needed to investigate the structure and function of TcTRP1 and TcTRP2.

In summary, C-terminal endogenous tagging with smHA tag epitopes allows the detection of low expressing channels in *T. cruzi* and will be useful for localization and for potential protein-protein interaction studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Juan Jose Cazzulo for antibody against serine carboxypeptidase, Thomas Seebeck for pMOTag plasmids, Loren I. Looger for the spaghetti monster containing plasmids, and Muthugapatti Kandasamy and the Biomedical Microscopy Core of the University of Georgia for the use of microscopes. This work was supported by U.S. National Institutes of Health grant AI107663 to RD.

LITERATURE CITED

- Bone GJ, Steinert M. Isotopes incorporated in the nucleic acids of *Trypanosoma mega*. *Nature*. 1956; 178:308–9. [PubMed: 13358724]
- Butt AH, Rasool N, Khan YD. A treatise to computational approaches towards prediction of membrane protein and its subtypes. *J Membr Biol*. 2017; 250:55–76. [PubMed: 27866233]
- Cruz-Bustos T, Potapenko E, Storey M, Docampo R. An intracellular ammonium transporter is necessary for replication, differentiation, and resistance to starvation and osmotic stress in *Trypanosoma cruzi*. *mSphere*. 2018a; 3:e00377–17. [PubMed: 29359189]
- Cruz-Bustos T, Ramakrishnan S, Cordeiro CD, Ahmed MA, Docampo R. A riboswitch-based inducible gene expression system for *Trypanosoma brucei*. *J Eukaryot Microbiol*. 2018b Dec 19.
- Di Paola S, Scotto-Rosato A, Medina DL. TRPML1: The Ca²⁺ retaker of the lysosome. *Cell Calcium*. 2018; 69:112–121. [PubMed: 28689729]
- Docampo R. Molecular parasitology in the 21st century. *Essays Biochem*. 2011; 51:1–13. [PubMed: 22023438]
- Dong XP, Cheng X, Mills E, Delling M, Wang F, Kurz T, Xu H. The type IV mucopolipidosis-associated protein TRPML1 is an endolysosomal iron release channel. *Nature*. 2008; 455:992–6. [PubMed: 18794901]
- Dong XP, Shen D, Wang X, Dawson T, Li X, Zhang Q, Cheng X, Zhang Y, Weisman LS, Delling M, Xu H. PI(3,5)P₂ controls membrane trafficking by direct activation of mucolipin Ca²⁺ release channels in the endolysosome. *Nat Commun*. 2010; 1:38. [PubMed: 20802798]
- Eichelsdoerfer JL, Evans JA, Slaugenhaupt SA, Cuajungco MP. Zinc dyshomeostasis is linked with the loss of mucopolipidosis IV-associated TRPML1 ion channel. *J Biol Chem*. 2010; 285:34304–8. [PubMed: 20864526]
- Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, Salazar GA, Tate J, Bateman A. The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res*. 2016; 44:D279–85. [PubMed: 26673716]
- Finn RD, Attwood TK, Babbitt PC, Bateman A, Bork P, Bridge AJ, Chang HY, Dosztanyi Z, El-Gebali S, Fraser M, Gough J, Haft D, Holliday GL, Huang H, Huang X, Letunic I, Lopez R, Lu S, Marchler-Bauer A, Mi H, Mistry J, Natale DA, Necci M, Nuka G, Orengo CA, Park Y, Pesceat S, Piovesan D, Potter SC, Rawlings ND, Redaschi N, Richardson L, Rivoire C, Sangrador-Vegas A, Sigrist C, Sillitoe I, Smithers B, Squizzato S, Sutton G, Thanki N, Thomas PD, Tosatto SC, Wu CH, Xenarios I, Yeh LS, Young SY, Mitchell AL. InterPro in 2017-beyond protein family and domain annotations. *Nucleic Acids Res*. 2017; 45:D190–D199. [PubMed: 27899635]

- Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R. A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res.* 2010; 38:W695–9. [PubMed: 20439314]
- Hortua-Triana M, Marquez-Nogueras K, Le C, Stasic AJ, Spiegel K, Sharma A, Li ZH, Moreno SNJ. High-performance probes for the localization of weakly expressed *Toxoplasma gondii* calcium related genes. *J Eukaryot Microbiol.* 2018 in press.
- Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, Pesseat S, Quinn AF, Sangrador-Vegas A, Scheremetjew M, Yong SY, Lopez R, Hunter S. InterProScan 5: genome-scale protein function classification. *Bioinformatics.* 2014; 30:1236–40. [PubMed: 24451626]
- Kall L, Krogh A, Sonnhammer EL. A combined transmembrane topology and signal peptide prediction method. *J Mol Biol.* 2004; 338:1027–36. [PubMed: 15111065]
- Lander N, Chiurillo MA, Docampo R. Genome editing by CRISPR/Cas9: a game change in the genetic manipulation of protists. *J Eukaryot Microbiol.* 2016a; 63:679–90. [PubMed: 27315329]
- Lander N, Li ZH, Niyogi S, Docampo R. CRISPR/Cas9-induced disruption of paraflagellar rod protein 1 and 2 genes in *Trypanosoma cruzi* reveals their role in flagellar attachment. *MBio.* 2015; 6:e01012. [PubMed: 26199333]
- Lander N, Chiurillo MA, Storey M, Vercesi AE, Docampo R. CRISPR/Cas9-mediated endogenous C-terminal tagging of *Trypanosoma cruzi* genes reveals the acidocalcisome localization of the inositol 1,4,5-trisphosphate receptor. *J Biol Chem.* 2016b; 291:25505–25515. [PubMed: 27793988]
- Oberholzer M, Morand S, Kunz S, Seebeck T. A vector series for rapid PCR-mediated C-terminal in situ tagging of *Trypanosoma brucei* genes. *Mol Biochem Parasitol.* 2006; 145:117–20. [PubMed: 16269191]
- Parussini F, Garcia M, Mucci J, Aguero F, Sanchez D, Hellman U, Aslund L, Cazzulo JJ. Characterization of a lysosomal serine carboxypeptidase from *Trypanosoma cruzi*. *Mol Biochem Parasitol.* 2003; 131:11–23. [PubMed: 12967708]
- Prole DL, Taylor CW. Identification of intracellular and plasma membrane calcium channel homologues in pathogenic parasites. *PLoS One.* 2011; 6:e26218. [PubMed: 22022573]
- Sant'Anna C, Parussini F, Lourenco D, de Souza W, Cazzulo JJ, Cunha-e-Silva NL. All *Trypanosoma cruzi* developmental forms present lysosome-related organelles. *Histochem Cell Biol.* 2008; 130:1187–98. [PubMed: 18696100]
- Scott DA, Docampo R, Dvorak JA, Shi S, Leapman RD. In situ compositional analysis of acidocalcisomes in *Trypanosoma cruzi*. *J Biol Chem.* 1997; 272:28020–28029. [PubMed: 9346954]
- Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol.* 2011; 7:539. [PubMed: 21988835]
- Soares Medeiros LC, South L, Peng D, Bustamante JM, Wang W, Bunkofski M, Perumal N, Sanchez-Valdez F, Tarleton RL. Rapid, selection-free, high-efficiency genome editing in protozoan parasites using CRISPR-Cas9 ribonucleoproteins. *MBio.* 2017; 8:e01788–17. [PubMed: 29114029]
- Taylor MC, McLatchie AP, Kelly JM. Evidence that transport of iron from the lysosome to the cytosol in African trypanosomes is mediated by a mucolipin orthologue. *Mol Microbiol.* 2013; 89:420–32. [PubMed: 23750752]
- Venkatachalam K, Montell C. TRP channels. *Annu Rev Biochem.* 2007; 76:387–417. [PubMed: 17579562]
- 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]

Figure 1.

(A) PCR analysis using gDNA isolated from WT, TcTRP1-3xHA (HA) and TcTRP1-SM (SM) cell lines. A DNA fragment was amplified in 3xHA-tagged and SM-tagged epimastigotes, whereas the band is absent in wild type (WT) cells. (B) Schematic representation of integration of 3xHA or SM, tubulin intergenic region (IgR), and antibiotic resistance gene at the 3'-end of *TcTRP1* by homologous recombination. Arrows indicate primers used for checking integration of donor DNA. (C) Western blot analyses of *TcTRP1*-3xHA and *TcTRP1*-SM expressing cell lines and WT epimastigotes as a control.

Anti-HA antibodies detect bands of 55 kDa and 85 KDa. Anti-tubulin antibody was used as loading control. Apparent molecular weights in kDa are on the left side. (D) Localization of TcTRP1 in epimastigotes. Endogenously-tagged TcTRP1-3xHA detected with anti-HA antibodies (*red*) co-localizes with anti-SCP antibodies (*green*). Endogenously-tagged TcTRP1-SM also co-localizes with anti-SCP. DIC, differential interference contrast microscopy. Merge in *yellow*. DAPI staining in *blue*. Bars = 5 μ m. (E) Anti-HA and anti-SM fluorescence in *TcTRP1-3xHA*- and *TcTRP1-SM*-expressing epimastigotes, respectively (means $\pm$ s.d., n = 3, **P* < 0.05, Student's t test). (F) PCR analysis using gDNA isolated from *WT*, *TcTRP2-3xHA*- and *TcTRP2-SM*-expressing cell lines. A DNA fragment was amplified in 3xHA-tagged and SM-tagged epimastigotes, whereas the band is absent in *WT* cells. The *WT* was from the same gel and is separated because a duplicate sample of *TcTRP1-SM* cell line that was in the original was deleted. (G) Schematic representation of integration of 3xHA or SM, IgR, and antibiotic resistance gene at 3'-end of *TcTRP2* by homologous recombination. Arrows indicate primers used for checking integration of donor DNA. (H) Western blot analyses of *TcTRP2-3xHA*- and *TcTRP2-SM*-expressing cell lines and *WT* epimastigotes as a control. Anti-HA antibodies detect bands of 90 kDa and a triplet of 130, 140, and 150 kDa. Anti-tubulin antibody was used as loading control. Apparent molecular weights in kDa are on the left side. (I) Localization of TcTRP2 in epimastigotes. TcTRP2-3xHA detected with anti-HA antibodies (*red*) partially co-localizes with anti-SCP (*green*). Endogenously-tagged TcTRP2-SM also co-localizes with anti-SCP. DIC, differential interference contrast microscopy. Merge in *yellow*. DAPI staining in *blue*. Bars = 5 μ m. (J) Anti-HA and anti-SM fluorescence in *TcTRP2-3xHA*- and *TcTRP2-SM*-expressing epimastigotes, respectively (means $\pm$ s.d., n = 3, **P* < 0.05, Student's t test).