CRISPR/Cas9-mediated endogenous C-terminal tagging of *Trypanosoma cruzi* genes reveals the acidocalcisome localization of the inositol-1,4,5-trisphosphate receptor

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Methods for genetic manipulation of *Trypanosoma cruzi*, the etiologic agent of Chagas disease, have been highly inefficient and no endogenous tagging of genes has been reported to date. We report here the use of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated gene 9) system for endogenously tagging genes in this parasite. The utility of the method was established by tagging genes encoding proteins of known localization such as the flagellar calcium binding protein (TcFCaBP), and the vacuolar proton pyrophosphatase (TcVP1), and two proteins of undefined or disputed localization, the mitochondrial calcium uniporter (TcMCU), and the inositol-1,4,5-trisphosphate receptor (TcIP3R). We confirmed the flagellar and acidocalcisome localization of TcFCaBP and TcVP1, by co-localization with antibodies to the flagellum and acidocalcisomes, respectively. As expected, TcMCU was co-localized with the voltage-dependent anion channel (VDAC) to the mitochondria. However, in contrast to previous reports and our own results using overexpressed TcIP3R, endogenously tagged TcIP3R showed co-localization with antibodies against VPI to acidocalcisomes. These results are also in agreement with our previous reports on the localization of this channel to acidocalcisomes of *T. brucei* and suggest that caution should be exercised when overexpression of tagged genes is done to localize proteins in *T. cruzi*.

The application of the CRISPR/Cas9 technology to the study of protist parasites has dramatically increased the tools available for their genetic manipulation (1). *Trypanosoma cruzi*, the etiologic agent of Chagas disease, which is a significant cause of morbidity and mortality from the South of the US to the South of Argentina and Chile, has been particularly refractory to genetic manipulation. However, the recent use of the CRISPR/Cas9 technology to knockdown or knockout genes (2,3) has revolutionized their study.

The localization of proteins is important to determine their cellular function and previous studies in *T. cruzi* have used either antibodies or gene tagging methods using vectors that overexpressed the proteins (4). While specific antibodies are useful to detect the endogenous proteins, it is not always possible to obtain them because either the proteins have low antigenicity or the antibodies cross-react with other proteins. Plasmids that enable the tagging of genes at their endogenous loci are not available for *T. cruzi* and a major drawback of the overexpression of tagged proteins is that the proteins of interest sometimes are retained in the endoplasmic reticulum (ER), or localize to other compartments.

Here we have adapted the CRISPR/Cas9 system to tag genes of *T. cruzi* at their endogenous loci and tested this system with two genes encoding proteins of well recognized localization (flagellar calcium binding protein or TcFCaBP and the acidocalcisome vacuolar proton pyrophosphatase or TcVP1) and two encoding proteins of undefined or disputed localization (mitochondrial calcium uniporter or TcMCU and inositol-1,4,5-trisphosphate receptor or TcIP3R). TcMCU was functionally characterized more than 27 years ago as the calcium channel that transports Ca2+ into the mitochondria of the parasites (5,6) and this finding was fundamental for the recent discovery of the gene encoding this channel in mammalian cells (7-9).
The channel is localized to the inner mitochondrial membrane of a variety of cells, including *T. brucei* (10). TcIP3R was reported to have endoplasmic reticulum (ER) localization in *T. cruzi* (11). However, the immunofluorescence evidence reported was disputed (12), as there was no clear reticular pattern or colocalization with a TcIP3R ER marker, TbbBiP, in the figures published (11). In addition, the *T. brucei* IP3R localized to the acidocalcisomes as demonstrated using antibodies against the endogenous tagged protein (13), specific antibodies against the protein (14), as well as proteomic and functional analyses (13,14). In this work we report the acidocalcisome localization of TcIP3R.

The use of the CRISPR/Cas9 system for C-terminal tagging of genes was recently reported for three parasites, *Toxoplasma gondii* (15), *Plasmodium yoelii* (16), and *Leishmania donovani* (17) but has not been previously used in *T. cruzi*. The availability of this technique for *T. cruzi* has great potential for the functional analysis of proteins in this parasite.

### Results

We first evaluated the endogenous C-terminal tagging method by introducing the epitope tag sequence into two different genes: the TcFCaBP gene and the TcVP1 gene. The proteins encoded by these genes are localized in well-defined organelles in trypanosomes: flagellum (18), and acidocalcisomes (19), respectively. Monoclonal and polyclonal antibodies recognizing these proteins are available, as well as genetic information about the proteins. For 3xHA C-terminal tagging we cotransfected a specific 3’end-sgRNA/Cas9/pTREX construct with a specific DNA donor molecule for each gene amplified from the pMOTag23M vector (Fig. 1A), while for 3xc-Myc C-terminal tagging we cotransfected the same 3’end-sgRNA/Cas9/pTREX constructs with a specific DNA donor molecule for each gene amplified from the pMOTag23M vector (Fig. 1B), as described under Experimental Procedures. We obtained G418/hygromycin or G418/puromycin resistant cell lines after 5 weeks under selective pressure. Transfectants were analyzed by PCR, using gDNA isolated from each one, and specific primer sets to distinguish between the wild type and the tagged cell lines.

Fig. 2A shows that TcVP1-3xHA transfectants were efficiently tagged at the endogenous locus, as the corresponding band amplified with a reverse primer annealing on the hygromycin marker is only present in the resistant parasites (lane HA) but absent in the wild type (WT) cells, which is the negative control of the reaction. We analyzed the TcVP1-3xHA transfectants by western blot, using commercial antibodies anti-HA tag, and a band of ~85 kDa was clearly detected on the transfectant but absent in the wild type cells (Fig. 2B). A similar band appears in both wild-type cells and TcVP1-3xHA transfectants when anti-TbVP1 antibodies were used (Fig. 2B). Immunofluorescence analysis (IFA) of the mutants verified the subcellular localization of the protein to the acidocalcisomes as it co-localizes with antibodies against TbVP1 (Fig. 2C), as expected (19). Similar results were obtained by 3xc-Myc C-terminal tagging using specific DNA donor molecules amplified from the pMOTag23M vector (Figs. 2D-F). Site-specific insertion of DNA donor cassettes at the 3’ end of TcVP1 gene was verified by cloning and sequencing PCR products amplified from gDNA extracted from TcVP1-3xHA and TcVP1-3x-Myc cell lines (Fig. 3A), confirming that the mechanism of homologous-directed DNA repair (HDR) took place in almost the entire population, and a tagging efficiency >95% was observed in both cell lines by IFA (data shown for TcVP1-3x-Myc, Fig. 3B). These results also indicate that it is feasible to use the intergenic tubulin region of *T. brucei* as trans-spooling signal for *T. cruzi*.

Using similar procedures we found that the TcFCaBP was efficiently tagged at the endogenous locus, as detected by PCR (Figs. 4A and 4D), western blot analyses (Figs. 4B and 4E) and IFA of epimastigotes generated using specific DNA donor molecules amplified from either the pMOTag-HX1-4H (Fig. 4C) or the pMOTag23M vector (Fig. 4F). TcFCaBP-3xHA and TcFCaBP-3x-Myc exclusively localize in the flagellum, the expected localization of this protein (18), as shown by their co-localization with monoclonal antibodies against TcFCaBP (Figs. 4C and 4F), which recognizes both the tagged and endogenous proteins by western blot analyses (Figs. 4B and 4E). In both cases, detection of the endogenous non-tagged TcFCaBP was much stronger than the tagged version of the protein. We attribute this result to the fact that TcFCaBP is encoded by three identical copies of the gene arranged in tandem in the *T. cruzi* genome, and probably not all of them were tagged. The localization of C-terminal tagged TcVP1 and TcFCaBP at the expected compartments indicate that the method used is appropriate to detect the native localization of proteins in *T. cruzi* and that the two vectors employed, one of them designed for endogenous tagging of genes in *T. brucei*, are adequate for this purpose.

We next investigated the localization of two proteins...
for which either no previous localization studies have been reported (TcMCU) (20) or for which its localization has been disputed (TcIP3R) (12). TcMCU is the T. cruzi orthologue of the recently discovered MCU from vertebrate cells (8,9), and of TbMCU (10). MCU localizes to the inner membrane of mitochondria in both vertebrate cells (8,9), and T. brucei (10), and is the sole responsible for mitochondrial Ca<sup>2+</sup> uptake in T. brucei (10). Functional studies done in T. cruzi clearly established the presence of MCU in these cells (5,6) and were important for the identification of the molecular nature of this channel in vertebrate cells (7). Using the same technique that we used to localize TcVP1 and TcFCaBP (see above) we found the TcMCU was tagged at the endogenous locus, as detected by PCR (Figs. 5A and 5D), western blot analyses (Figs. 5B and 5E) and IFA of cells obtained using specific DNA donor molecules amplified from either the pMOTag-HX1-4H (Fig. 5C) or the pMOTag23M vector (Fig. 5F). TcMCU co-localized with the mitochondrial voltage-dependent anion channel (VDAC, Figs. 5C and 5F), as expected.

Before doing endogenous tagging of TcIP3R we overexpressed the gene with an HA epitope tag (TcIP3R-HA-OE) to investigate the localization of the overexpressed protein. Fig. 6A shows the western blot analysis of lysates from wild-type (WT) and TcIP3R-HA-OE epimastigotes (IP3R-HA) incubated with anti-HA antibodies showing that transfected cells express the tagged protein of the expected size (∼340 kDa). Fig. 6B shows that the overexpressed protein does not co-localize with the acidocalcisome marker TcVP1, as detected with antibodies against HA and TbVP1, respectively. However, TcIP3R-HA-OE cells shows the same perinuclear and reticular localization pattern as BiP, an ER marker (21), as detected with antibodies against HA and TbBiP. Note that although the same distribution pattern is observed for both proteins their localization is in general not superimposable. This is probably due to the membrane localization of TcIP3R-HA-OE, the intra-ER localization of the soluble BiP, and the fact that these images were deconvolved to eliminate background fluorescence.

Fig. 7 shows the efficient tagging of TcIP3R at the endogenous locus, as detected by PCR (Figs. 7A and 7E), western blot analyses (Figs. 7B and 7F) and IFA of tagged cell lines generated using specific DNA donor molecules amplified from either the pMOTag-HX1-4H (Figs. 7C and 7D) or the pMOTag23M vector (Figs. 7G and 7H). TcIP3R-3xHA and TcIP3R-3xc-Myc localize to the acidocalcisomes, as previously described in T. brucei (13) and shown by the co-localization of anti-HA and anti-c-Myc antibodies with VP1 (Figs. 7C and 7G). The anti-HA and anti-c-Myc antibodies recognize the tagged proteins but not the endogenous IP3R in the WT by western blot analyses (Figs. 7B and 7F). Figs. 7D and 7H show that there is no significant co-localization with the reticular distribution of TbBiP antibodies in the ER. Some co-localization with TbBiP antibodies, especially using TcIP3R-3xc-Myc was also detected (Fig. 7H) and could correspond to the site of synthesis of the TcIP3R in the ER.

Discussion

Our work demonstrates that the use of the CRISPR/Cas9 system in T. cruzi is not limited to loss-of-function studies (gene deletion/disruption/mutation) (2,3) but could be used for C-terminal gene tagging. As proof-of-concept of the methodology employed we confirmed the localization of TcVP1 and TcFCaBP to the acidocalcisomes and flagellum of the parasite, respectively. To our knowledge, this is the first report of endogenous tagging of proteins in T. cruzi. We also report the mitochondrial localization of TcMCU, the previously identified pore of the mitochondrial Ca<sup>2+</sup> uniporter complex (22). In addition, we report the acidocalcisome localization of TcIP3R.

We previously reported in T. cruzi the HDR mechanism for double strand break repair in CRISPR/Cas9-induced PFR2 knockout cell line (3). In that work we used a DNA donor molecule with 100-nt homology regions to induce DNA repair by homologous recombination in this organism, generating a homogeneous population where 100% cells exhibited gene disruption. Now, by providing a DNA donor template for CRISPR/Cas9-mediated gene tagging we confirmed the high efficiency of this mechanism, as no other DNA repair mechanism was detected by sequencing in TcVP1-3xHA and TcVP1-3xc-Myc homogeneously tagged populations (Fig. 3A). High efficiency (>95%) gene tagging was observed in all tagged cell lines generated in this study, using DNA donor templates containing 100-nt homology arms, which makes this methodology a promising tool for cellular localization studies and immunoprecipitation assays.

Although most vertebrate IP3Rs reside in ER membranes, IP3 can stimulate Ca<sup>2+</sup> release from the Golgi complex (23), the nucleus (24), and the secretory granules (25) of mammalian cells. IP3Rs can also be targeted to the plasma membrane, where they are important for Ca<sup>2+</sup> entry (26). Secretory granules of a
variety of cells (27-31) were reported to possess IP₃Rs, although this was challenged (32). An IP₃R localizes to the contractile vacuole in *Paramecium tetraurelia*, as detected with specific antibodies (33). The acidocalcisome localization of TbIP₃R in *T. brucei* was demonstrated by endogenous gene tagging (13), by studies using specific antibodies (14), and by proteomic (14) and functional (13) studies. Interestingly, when TbIP₃R (13) or the IP₃Rs of *Capsaspora owczarzaki* (another protist in which biochemical characterization of the channel was done (34)) are expressed in DT40-3KO cells (chicken lymphocytes in which the three vertebrate IP₃R have been knocked out), the proteins localize to the ER.

It was therefore puzzling that in the related trypanosomatid *T. cruzi* the IP₃R had an ER localization, as studied in epimastigotes overexpressing the channel tagged with GFP (11). This was also against our proteomic analysis of acidocalcisomes of *T. cruzi* (unpublished results), which supported the acidocalcisome localization. It has been reported that when overexpressed, membrane-targeted GFP fusion proteins have a propensity to form organelle aggregates that may lead to misinterpretations of sorting pathways of trafficked proteins (35). We found that this was indeed the case with the TcIP₃R. Overexpression of TcIP₃R resulted in the same pattern of perinuclear and reticular localization than that of the ER marker BiP. However, endogenous gene tagging of TcIP₃R using CRISPR/Cas9 revealed the acidocalcisome localization of the channel. The presence of a Ca²⁺ uptake pump (Ca²⁺-ATPase) (36) and a Ca²⁺ release channel (TcIP₃R, this work) in acidocalcisomes of *T. cruzi* suggest an important role of the organelles in Ca²⁺ signaling. The results also indicate that caution should be exercised when overexpression of tagged genes is done to localize proteins in *T. cruzi*.

In summary the tools developed in this work will enable rapid endogenous gene tagging in *T. cruzi*, allow to establish the localization of proteins and to gain insight into their function. The molecular tools available in *T. cruzi* have lagged behind those developed for *T. brucei* (4). Our results indicate that it is possible to use the intergenic tubulin region of *T. brucei* as transsplicing signal for *T. cruzi*, which expands the molecular toolbox available for *T. cruzi*, as pMOTag vectors (37) developed for C-terminal tagging in *T. brucei* could be also used for *T. cruzi*. The method developed in this work will facilitate the functional analysis of genes in *T. cruzi* as well as physiological studies allowing the identification of targets for drugs, diagnostics, and vaccines.

**Experimental Procedures**

*Chemicals and Reagents-* Hygromycin, blasticidin S HCl, BenchMark prestained protein ladder, BenchMark protein ladder, Alexa-conjugated secondary antibodies, and HRP-conjugated secondary antibodies were purchased from Life Technologies (Grand Island, NY). Benzonase® nuclease was from Novagen (EMD Millipore, Billerica, MA). GoTaq DNA polymerase, pGEM-T easy Vector System and T4 DNA ligase were from Promega (Madison, WI). Antarctic phosphatase, restriction enzymes and Q5® High-Fidelity DNA Polymerase were from New England Biolabs (Ipswich, MA). Fluoromount-G® was from SouthernBiotech (Birmingham, AL). Anti-HA high affinity rat monoclonal antibody (clone 3F10) was purchased from Roche (Roche Applied Science, Mannheim, Germany). Rabbit antibody against *T. brucei* vacuolar H⁺-pyrophosphatase (TbV1) (38) was from Dr. Norbert Bakalara (Ecole Nationale Supérieure de Chimie de Montpellier, Montpellier, France). Monoclonal antibody against FCaBP was from Dr. David Engman (Northwestern University, Evanston, IL). Polyclonal antibody against TbVDAC (39) was from Dr. Minu Chaudhuri (Meharry Medical College, TN). Polyclonal antibody against TbBiP (21) was from Dr. Jay Bangs (State University of New York at Buffalo, NY). The pMOTag vectors (37) were from Dr. Thomas Seebeck (University of Bern, Bern, Switzerland). DNA oligonucleotides were purchased from Exxtend Biotecnologia Ltda. (Campinas, Brazil). Pierce BCA protein assay and HA epitope tag monoclonal antibody (clone 2-2.2.14) were from Thermo Fisher Scientific Inc. (Rockford, IL). Rabbit anti-HA polyclonal antibody (Y-11), anti-c-Myc monoclonal antibody (clone 9E10) and rabbit anti-c-Myc polyclonal antibody (N-262) were from Santa Cruz Biotechnology (Dallas, TX). Anti-tubulin monoclonal antibody, puromycin, G418, mammalian cell protease inhibitor cocktail (Sigma P8340), other protease inhibitors, and all other reagents of analytical grade were from Sigma (St. Louis, MO).

*Cell Culture-* *T. cruzi* Y strain epimastigotes were cultured in liver infusion tryptose (LIT) medium containing 10% heat inactivated fetal bovine serum (FBS) at 28°C (40). CRISPR/Cas9 mutant cell lines were maintained in medium containing 250 µg/ml G418 and 5 µg/ml puromycin or 350 µg/ml hygromycin. We determined the growth rate of epimastigotes by counting cells in a Neubauer chamber.
Endogenous C-terminal tagging by CRISPR/Cas9-To achieve the C-terminal tagging of endogenous proteins we used the Cas9/pTREX-n vector we developed for *T. cruzi* (3) to clone a specific single guide RNA (sgRNA) sequence targeting the 3’ end of four different genes: *TcVP1* (Gene ID TcCLB.510773.20), *TcFCaBP* (TcCLB.509391.20), *TcMCU* (TcCLB.503893.120), and *TcIP3R* (*T. cruzi* Esmeraldo strain, contig KB205149.1, nt 21468 to 30512), encoding for the *T. cruzi* vacuolar proton pyrophosphatase, flagellar calcium binding protein, mitochondrial calcium uniporter, and inositol-1,4,5-trisphosphate receptor, respectively. Gene IDs are from the TryptDB (www.tritrypdb.org). Each one of these 4 constructs (3’end-sgRNA/Cas9/pTREX-n), together with DNA donor cassettes to induce homology directed repair, were used to co-transfect *T. cruzi* epimastigotes and to insert a specific tag sequence (3xHA tag or 3xc-Myc tag) at the 3’ end of each gene.

sgRNA targeting the 3’ end of these genes were designed to induce the double strand break by Cas9 nuclease downstream their stop codons. Chimeric sgRNAs were obtained by PCR from plasmid pUC_sgRNA as previously described (3) using specific oligonucleotides (Table 1, primers 1 to 5), which include a BamHI restriction site, the 20-nt specific protospacer region, and a 20-nt sequence that anneals to the sgRNA backbone. Subsequently these sgRNAs were cloned into Cas9/pTREX-n vector through BamHI site. To avoid Cas9 off-targeting protospacers were analyzed with ProtoMatch 1.0 script (15).

For the generation of a DNA donor cassette (DNA template to induce homologous-directed DNA repair) containing the tag sequence and a marker for antibiotic resistance, we used a modified version of the pMOTag4H vector (37), where the *T. brucei* tubulin intergenic region for trans-splicing was replaced by the HX1 *T. cruzi* trans-splicing signal present in the pTREX vector (41). The HX1 fragment was amplified using primers 6 and 7 from Table 1 and cloned into pMOTag4H vector by SalI/HindIII restriction sites. We then amplified fragment C was cloned into pET-32 EK/LIC vector (Novagen) (N-TcIP3R/pET-32) by XbaI and BglII restriction sites. Then, amplified fragment N was cloned into pET-32 EK/LIC vector (Novagen) (N-TcIP3R/pET-32) by XbaI/BglII for N-terminal region (N), and primer set Fw_TcIP3R-BglII/Rv_TcIP3R-HA-BglII for C-terminal region (C) (Table 1, primers 23 to 26), using *T. cruzi* Y strain gDNA as template. The amplified fragment N was cloned into pET-32 EK/LIC vector (Novagen) (N-TcIP3R/pET-32) by XbaI and BglII restriction sites. Then, amplified fragment C was cloned by BglIII into plasmid N-TcIP3R/pET-32, previously treated with Antarctic phosphatase, to obtain the TcIP3R/pET-32 plasmid. Next, the full sequence of TcIP3R-HA was excised with XbaI from TcIP3R/pET-32 plasmid and subcloned into dephosphorylated pTREX-n vector by XbaI to generate the TcIP3R-HA-OE/pTREX-n plasmid. Insert orientation was determined by PCR and sequencing.

**Cell Transfections**-Transfections were performed as previously described (3). Briefly, *T. cruzi* Y strain epimastigotes in early exponential phase (4 × 10^7 cells) were washed with phosphate-buffered saline (PBS), pH 7.4, at room temperature (RT) and transfected in ice-cold CytoMix (25 mM Hepes, 120 mM KCl, 0.15 mM CaCl_2, 10 mM K_2HPO_4, 2 mM EGTA, 5 mM MgCl_2, 0.5% glucose, 100 µg/ml bovine serum albumin [BSA], 1 mM hypoxanthine [pH 7.6]) containing 25 µg of each plasmid construct and 25 µg of donor DNA in 4-mm electroporation cuvettes with three pulses (1,500 V, 25 µF) delivered by a Gene Pulser II (Bio-Rad). Stable cell lines were established and maintained under drug resistance.
selection (250 µg/ml G418 and 350 µg/ml hygromycin or 5 µg/ml puromycin). Transfectant epimastigotes were cultured in LIT medium supplemented with 20% heat-inactivated FBS until stable cell lines were obtained.

**PCR analysis of transfected epimastigotes**—Genomic DNA of double-resistant transfectants was used as template in PCR reactions to verify the integration of the DNA donor molecules into the 3′ end of the tagged genes. In each PCR reaction was included a genespecific forward primer (Table 1, primers 16-19) and a reverse primer annealing at 3′end of the antibiotic marker present in the donor DNA (Table 1, primers 20 and 21). PCR conditions in a 25 µl reaction volume using GoTaq DNA polymerase with ~20 ng gDNA were as follows: 35 cycles of 95°C for 20 s, 57°C for 30 s and 72°C for 2 min 20 s followed by a final extension 72°C for 10 min. *TcVP1* site-directed tagging at nucleotide level was confirmed by sequencing several clones of PCR products obtained with primers 17 and 22 (Table 1) cloned into pGEM-T easy vector.

**Western blot analysis**—Western blot analyses were performed using standard procedures used in our laboratory (42,43). Parental and mutant cell lines were harvested separately. Parasites were washed twice in 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 mammalian cell protease inhibitor mixture (diluted 1:250), 1 mM phenylmethylsulfonyl fluoride, 2.5 mM tosyl phenylalanyl chloromethyl ketone (TPCK), 100 µM *N*-trans-epoxysuccinyl-L-leucine 4-guanidinobutylamide (E64), and Benzonase Nuclease (25 U/ml of culture). The cells were then incubated for 1 h on ice. Cell lysis was verified under a light microscope, and protein concentration was determined by a bicinchoninic acid protein assay. Thirty micrograms of protein from each cell lysate were mixed with 6x Laemmli sample buffer (125 mM Tris-HCl, pH 7, 10% (w/v) β-mercaptoethanol, 20% (v/v) glycerol, 4.0% (w/v) SDS, 4.0% (w/v) bromophenol blue) before application to 10%, 12%, or 6-12% (gradient) SDS-polyacrylamide gels (depending on antigen size) without previous boiling. Separated proteins were transferred onto nitrocellulose membranes (Bio-Rad) with a Bio-Rad Trans-blot apparatus. Membranes were blocked with 5% nonfat dried skim milk in PBS-T (PBS containing 0.1% vol/vol Tween 20) overnight at 4°C. Next, blots were incubated for 1 h at RT with a primary antibody, i.e., monoclonal anti-TcFCaBP antibody (1:100 dilution), polyclonal rabbit anti-TbVP1 antibody (1:2,000), monoclonal anti-HA-Tag (1:5,000), monoclonal anti-c-Myc-tag (1:100), and monoclonal anti-tubulin (1:40,000). After three washes with PBS-T, blots were incubated with the secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG, HRP-conjugated antibody, diluted 1:10,000). Membranes were washed three times with PBS-T, and Western blot images were obtained and processed with a C-DiGit Blot Scanner (LI-COR Biosciences).

**Immunofluorescence Analysis**—Epimastigotes were washed with PBS and fixed with 4% paraformaldehyde in PBS for 1 h at RT. Cells were allowed to adhere to poly-L-lysine-coated coverslips and then permeabilized for 5 min with 0.1% Triton X-100. Permeabilized cells were blocked with PBS containing 3% BSA, 1% fish gelatin, 50 mM NH₄Cl, and 5% goat serum overnight at 4°C. Then, cells were incubated with a primary antibody (monoclonal anti-TbFCaBP [1:10], polyclonal rabbit anti-TbVP1 [1:250], monoclonal anti-HA-Tag [1:500], rat anti-HA-Tag [1:10], monoclonal anti-c-Myc-tag [1:10], rabbit anti-c-Myc-tag [1:50], rabbit anti-TbBiP [1:50], rabbit anti-TbVDAC [1:200]) diluted in 1% BSA in PBS (pH 8.0) for 1 h at RT. Cells were washed three times with 1% BSA in PBS (pH 8.0), and then incubated for 1 h at RT in the dark with Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 546-conjugated goat anti-rabbit, or Alexa Fluor 546-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (1:1,000). Following incubation with the secondary antibody, cells were washed and 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 in the absence of a primary antibody. 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 charged-coupled device camera driven by DeltaVision software (Applied Precision, Issaquah, WA) and deconvolved for 15 cycles using Softwarx deconvolution software (Fig. 6) or with a confocal microscope Leica TCS SP5 II, with a 100X objective (1.44 aperture) under nonsaturating conditions, that uses photomultiplier tubes (PMTs) for detection of emission, and LAS AF software (Leica, Wetzlar, Germany) for acquisition and processing of digital images (Figs. 2-5, and 7).

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Conflict of interest- The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions—N.L., and M.A.C. designed and conducted the experiments and analyzed the data. R.D. wrote the majority of the manuscript, with specific sections contributed by N.L., and M.A.C. R.D. and A.E.V. supervised the work and contributed to the analysis of experiments. M.S. performed the IFA experiments with TcIP3R-HA-OE cells.

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**FOOTNOTES**

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FIGURE LEGENDS

FIGURE 1. Schematic representation of strategies used to generate endogenous C-terminal tagging in T. cruzi. A, i) pMOTag-HX1-4H vector map. The T. cruzi HX1 trans-splicing signal is located between the 3xHA tag sequence and the gene that confers resistance to hygromycin (Hygro®). HR1 Fw and HR2 Rv ultramers indicate oligonucleotides used to amplify DNA donor cassette. The annealing regions for ultramers to pMOTag-HX1-4H and genomic DNA (gDNA) are indicated in black and blue, respectively. ii) A double-stranded gDNA break was produced by Cas9 targeted by the sgRNA both expressed from 3’end-sgRNA/Cas9/pTREX plasmid downstream the STOP codon of the gene of interest (GOI) in the endogenous locus. Homologous directed repair was induced co-transfecting epimastigotes with the DNA donor cassette, containing homologous regions to the GOI 3’ end (blue) and to the GOI 3’UTR (light blue). iii) Integration of 3xHA and antibiotic resistance gene at 3’end of GOI by homologous recombination. Arrows indicate primers used for checking integration of donor DNA. B, i) pMOTag23M vector map. The 3xc-Myc tag sequence and the puromycin resistance gene (Puro (R)) are separated by the T. brucei tubulin intergenic region (Tigr). The rest of i) ii) and iii) description is similar to that panel A. Hygro, hygromycin resistance gene; Puro, puromycin resistance gene; UTR, 5’and 3’untranslated regions; ATG, start codon.

FIGURE 2. TcVP1 endogenous C-terminal tagging. A, PCR analysis using gDNA isolated from wild type (WT) and TcVP1-3xHA cell lines. A DNA fragment was amplified in 3xHA-tagged epimastigotes (indicated with arrow), while the band is absent in WT. B, Western blot analysis of WT and TcVP1-3xHA cell lines. Anti-HA antibodies detect TcVP1-3xHA (expected size 89 kDa) and anti-TbVP1 antibodies detect endogenous TcVP1 (85 kDa). Anti-α-tubulin antibody was used as a loading control. Antibodies are indicated on the right side of the blots and molecular weights on the left side. C, Fluorescence microscopy of TcVP1-3xHA epimastigotes indicates localization of the endogenous tagged protein to acidocalcisomes. TcVP1-3xHA was detected with monoclonal anti-HA antibodies (green) or with polyclonal anti-TbVtc4 antibodies (red). D, PCR analysis of TcVP1-3xc-Myc epimastigotes. A DNA fragment was amplified in c-Myc-tagged epimastigotes (indicated with arrow), while the band is absent in WT cells. E, Western blot analysis of WT and TcVP1-3xc-Myc cell lines. Anti-c-Myc antibodies detect TcVP1-3xc-Myc (expected size 91 kDa). Anti-TbVP1 antibodies detect endogenous TcVP1 (85 kD). F, Fluorescence microscopy of TcVP1-3xc-Myc epimastigotes indicates localization of the endogenous tagged protein to acidocalcisomes. TcVP1-3xc-Myc was detected with monoclonal anti-c-Myc antibodies (green) or with polyclonal anti-TbVP1 antibodies (red). The merge shows co-localization in yellow. Differential interference contrast (DIC) images are shown on the left panel. Nucleus and kinetoplast were labeled with DAPI (blue). Bars = 10 µm.

FIGURE 3. Sequence analysis of TcVP1 locus and tagging efficiency. A, i) nucleotide sequence of wt TcVP1 showing the Cas9-targeted cut site (vertical arrow) downstream the stop codon (boxed). Homologous template sequences included in ultramers to generate the DNA donor for HDR are shown as horizontal lines. Schematic representation of wt TcVP1 is shown on top of the panel. ii and iii) Nucleotide sequences of 3xHA and 3xc-Myc tagged TcVP1 loci at the repaired region after Cas9-targeted double strand break in homogenously tagged populations. Schematic representation of tagged TcVP1 locus is shown on top of each panel. At the bottom of each panel the nucleotide sequence between the left and right arms of the homologous regions is shown. Colored regions indicated parental and tagged inserted sequences derived from pMOTag-HX1-4H (ii) and pMOTag-23M (iii). Dashed lines above traces indicate the nucleotide sequence of each vector included in the donor DNA, located upstream and downstream the specific tag and the resistance marker, respectively. A continuous line under nucleotide sequence indicates the inserted region in each tagged cell line. Stop codons of antibiotic resistance genes are shown in squared boxes. The asterisk indicates a nucleotide difference between wt (Y strain) and tagged cell lines, because the sequence of T. cruzi CL Brener Esmeraldo-like haplotype was used to design the ultramers for DNA donor amplification. B, Immunofluorescence microscopy of TcVP1-3xc-Myc epimastigotes using monoclonal anti-c-Myc antibodies (green) and polyclonal anti-TbVP1 antibodies (red). Differential interference contrast (DIC) merged image is shown on the right panel. The image shows parasites observed in an entire field. Scale bar = 10 µm.
FIGURE 4. **TcFCaBP endogenous C-terminal tagging.** A, PCR analysis using gDNA isolated from wild type (WT) and TcFCaBP-3xHA cell lines. A DNA fragment was amplified in 3xHA-tagged epimastigotes (indicated with arrow), while the band is absent in WT. B, Western blot analysis of WT and TcFCaBP-3xHA cell lines. Anti-HA antibodies detect TcFCaBP-3xHA (predicted size 28 kDa) and anti-FCaBP antibodies detect both, endogenous (24 kDa) and tagged (28 kDa) TcFCaBP. Anti-α-tubulin antibody was used as a loading control. Antibodies are indicated on the right side of the blots and molecular weights on the left side. C, Fluorescence microscopy of TcFCaBP-3xHA epimastigotes indicates localization of the endogenous tagged protein to flagellum. TcFCaBP-3xHA was detected with rat anti-HA antibodies (green) or with monoclonal anti-FCaBP antibodies (red). D, PCR analysis of TcFCaBP-3x-Myc epimastigotes. A DNA fragment was amplified in c-Myc-tagged epimastigotes (indicated with arrow), while the band is absent in WT cells. E, Western blot analysis of WT and TcFCaBP-3xc-Myc cell lines. Anti-c-Myc antibodies detect TcFCaBP-3xc-Myc (predicted size 30 kDa). Anti-FCaBP antibodies detect endogenous (24 kDa) and c-Myc-tagged (30 kDa) TcFCaBP. F, Fluorescence microscopy of TcFCaBP-3xc-Myc epimastigotes indicates localization of the endogenous tagged protein to flagellum. TcFCaBP-3xc-Myc was detected with rabbit anti-c-Myc antibodies (green) or with monoclonal anti-TcFCaBP antibodies (red). The merge shows co-localization in yellow. Differential interference contrast (DIC) images are shown on the left panel. Nucleus and kinetoplast were labeled with DAPI (blue). Bars = 10 µm.

FIGURE 5. **TcMCU endogenous C-terminal tagging.** A, PCR analysis using gDNA isolated from wild type (WT) and TcMCU-3xHA cell lines. A DNA fragment was amplified in 3xHA-tagged epimastigotes (indicated with arrow), while the band is absent in WT. B, Western blot analysis of WT and TcMCU-3xHA cell lines. Anti-HA antibodies detect TcMCU-3xHA (predicted size 35 kDa) while the band is absent in WT parasites. Anti-α-tubulin antibody was used as a loading control. Antibodies are indicated on the right side of the blots and molecular weights on the left side. C, Fluorescence microscopy of TcMCU-3xHA epimastigotes indicates localization of the endogenous tagged protein to mitochondria. TcMCU-3xHA was detected with monoclonal anti-HA antibodies (green). Polyclonal antibodies anti-TbVDAC were used to label mitochondria (red). The merge shows co-localization in yellow. D, PCR analysis of TcMCU-3xc-Myc epimastigotes. A DNA fragment was amplified in c-Myc-tagged epimastigotes (indicated with arrow), while the band is absent in WT cells. E, Western blot analysis of WT and TcMCU-3xc-Myc cell lines. Anti-c-Myc antibodies detect TcMCU-3xc-Myc (predicted size 37 kDa) while the band is absent in WT epimastigotes. F, Fluorescence microscopy of TcMCU-3xc-Myc epimastigotes indicates localization of the endogenous tagged protein to mitochondria. TcMCU-3xc-Myc was detected with monoclonal anti-c-Myc antibody (green) or with polyclonal anti-TbVDAC antibodies (red). The merge shows co-localization in yellow. Differential interference contrast (DIC) images are shown on the left panel. Nucleus and kinetoplast were labeled with DAPI (blue). Bars = 10 µm.

FIGURE 6. **TcIP3R-HA overexpression exhibits an ER localization pattern.** A, Western blot analysis of WT and TcIP3R-3HA-OE cell lines. Anti-HA antibodies detect TcIP3R-HA (predicted size 340 kDa) while the band is absent in WT parasites. Anti-α-tubulin antibody was used as a loading control. Antibodies are indicated on the right side of the blots and molecular weights on the left side. B, Fluorescence microscopy of TcIP3R-3HA-OE epimastigotes indicates an ER localization pattern of the overexpressed tagged protein. TcIP3R-HA-OE was detected with rat anti-HA antibodies (1:100, green). Rabbit antibodies anti-TbVP1 (1:500) and anti-TbBiP (1:500) were used to label acidocalcisomes and ER, respectively (red). The merge images are shown on the right panel. Differential interference contrast (DIC) images are shown on the left panel. Nucleus and kinetoplast were labeled with DAPI (blue). Bars = 10 µm.

FIGURE 7. **Endogenous tagged TcIP3R localizes to acidocalcisomes.** A, PCR analysis using gDNA isolated from wild type (WT) and TcIP3R-3xHA cell lines. A DNA fragment was amplified in 3xHA-tagged epimastigotes (indicated with arrow), while the band is absent in WT. B, Western blot analysis of WT and TcIP3R-3xHA cell lines. Anti-HA antibodies detect TcIP3R-3xHA (predicted size 342 kDa) while the band is absent in WT parasites. Anti-α-tubulin antibody was used as a loading control. Antibodies are indicated on the right side of the blots and molecular weights on the left side. C, Fluorescence microscopy of TcIP3R-3xHA epimastigotes indicates localization of the endogenous tagged protein to acidocalcisomes. TcIP3R-3xHA was detected with monoclonal anti-HA antibodies (green). Polyclonal antibodies anti-TbVP1 (C) and anti-TbBiP (D) were used to label acidocalcisomes and ER, respectively (red). The merge shows co-localization with TbVP1 in yellow. E, PCR analysis of TcIP3R-3xc-Myc epimastigotes. A DNA fragment was amplified in c-Myc-tagged epimastigotes (indicated with arrow), while the band is absent in WT cells. F, Western blot analysis of
WT and TcIP\textsubscript{R}-3xc-Myc cell lines. Anti-c-Myc antibodies detect TcIP\textsubscript{R}-3xc-Myc (predicted size 344 kDa) while the band is absent in WT epimastigotes. G, H, Fluorescence microscopy of TcMCU-3xc-Myc epimastigotes indicates localization of the endogenous tagged protein to acidocalcisomes. TcMCU-3xc-Myc was detected with monoclonal anti-c-Myc antibody (green). Polyclonal antibodies anti-TbVP1 ($G$) and anti-TbBiP ($H$) were used to label acidocalcisomes and ER, respectively (red). The merge shows co-localization with TcVP1 in yellow. Differential interference contrast (DIC) images are shown on the left panel. Nucleus and kinetoplast were labeled with DAPI (blue). Bars = 10 µm.
| N° | Primer          | Sequence (5’ → 3’)                                      |
|----|----------------|--------------------------------------------------------|
| 1  | Fw sgRNA_FCaBP | GATCGGATCCGGAGAGCGCTGAAGCTCGgttttagagctagaaatagc      |
| 2  | Fw sgRNA_VP1   | GATCGGATCCAGTGAACGCCTGAAGCCGGgttttagagctagaaatagc    |
| 3  | Fw sgRNA_MCU   | GATCGGATCCGATGAAGGGACCTAAGCAGgttttagagctagaaatagc   |
| 4  | Fw sgRNA_IP3R  | GATCGGATCTAAAAGAATATTGCAATGCgttttagagctagaaatagc    |
| 5  | Rv sgRNA_all   | CAGTGATCCaaaaaagcaccgactcggtg                         |
| 6  | Fw_HX1-SalI    | ATCGGTGACAACGAGTTTCTTCAAAAATATGCAGC                  |
| 7  | Rv_HX1-HindIII | GATCAAGCTTTAGACAACTATATAGAGC                         |
| 8  | Fw_FCaBP_Ctag_ultramer | GAACGGCACTGGGTCCGTGACGGAGTTTGCTGCTGGGTTGCTTCTGCCAGTCAAAACTGGACGC CGACGGCGACCGGGAGAGCCGGgttaccggggccccccctcgag |
| 9  | Rv_FCaBP_Ctag_ultramer | CATAAAGTGGAATGTGCTTCCGCACAGCAAAAACAAATTGCGCTGCGCAACAGACCGGTTTT ACATTGGCAACCGGGCGGACGTACTCCCGGGtgtgcggcccctattgtag                      |
| 10 | Fw_VP1_Ctag_ultramer | CGCGCTGAACATTTCTGATCAAACGATGGCCATCTTCTGGTGCTTCTTCTGGCGCTTCTTCTGAGTC ACAGCTGGCGCATTCTCAGGGACGGTACATTGAGgttaccggggccccccctcgag             |
| 11 | Rv_VP1_Ctag_ultramer | AGGCAAAAACACGCGGGAGAAAAATAATGGCGACAGGCAAAATATAAAAACAT AAAAATAAAAAATAAAAAACACCGTTCTGTggccggcccccccttaggtgtag                      |
| 12 | Fw_MCU_Ctag_ultramer | TCCTCCTGCTGGTTTGTAGTTGGGAATAATATAGCGGCCTCTGCCAAAAATGTGAGTAGGAACGAAGAAAT GTTGAATAAGATCAAGGATGATGAAAGAACACgttaccggggccccccctcgag           |
| 13 | Rv_MCU_Ctag_ultramer | ATGGTCAGGATGGAGCGATGGAAATAAAAACAAATAAATAAGACAGGGTTTGGCATTCACAGCAAGAT |
ATAAACATATGCATGCACGCCCCAAAGTTTTTCCT
tggccggcgtcttagactagtgat

Fw_IP3R-Ctag_ultramer
CACACCGAATGACCAGGCCATCTACCACCACCGACAAGCTTGGGTAGCAGAGGGTCCGGTGA
ACTGCGACTTCCTCCATGGGAGAAGACAGCAAAAAAT
tggccggcgtcttagactagtgat

Rv_IP3R-Ctag_ultramer
CCTCCCCAAATAGACACACACACACACACACACAGACAGACAAAGAGAACAGAGATAAAAATCCT
CACAAGAAAAATACATAAAAGTTTTCTTCTCCTGCA
tggccggcgtcttagactagtgat

Fw_FCaBP_Ctag_check
ATGGGTGCTTTGTTGGTCGAA

Fw_VP1_Ctag_check
CCACGAACATCATCTACGGC

Fw_MCU_Ctag_check
GCAATGCTGCATATGTGTATATGG

Fw_IP3R_Ctag_check
GCACAGAAAGATGTCTCAGGG

Rv_Puro_Ctag_check
TCAGGCACCGGGCTTGCGGG

Rv_Hygro_Ctag_check
CTATTCCTTTGCCCTCGGAC

Rv_VP1_Ctag_Check
GTCGTTTTGTCTTGCACG

Fw_TcIP3R-XbaI
CATCTCTAGATGGATCGAAAGCAACGC

Rv_TcIP3R-BglII
TCATAGATCTGAGTCGGAGGAGCAAC

Fw_TcIP3R-BglII
GCTCAGATCTGAGTCGGAGGAGCAAC

Rv_TcIP3R-HA-XbaI-BglII
GATCAGTCTAGATGGATCGAAAGCAACGC

Bold uppercase: specific protospearc; italic underlined uppercase: restriction site; lower case: sgRNA annealing region; bold underlined uppercase: gene-specific homologous region; italic lower case: pMOTag vector annealing region, italic bold uppercase: stop codon; bold double-underlined uppercase: HA tag sequence.
Figure 1

A

\[ \text{pMOTag-HX1-4H} \]

- HR1
- Fw ulramer
- HR2
- Rv ulramer

ii)

\[ \text{5'UTR} \quad \text{GOI} \quad \text{3'UTR} \quad \text{gDNA} \]

iii)

\[ \text{5'UTR} \quad \text{GOI} \quad \text{3'UTR} \]

- Gene-specific sgRNA
- Fw primer
- Rv primer

B

\[ \text{pMOTag-23M} \]

- HR1
- Fw ulramer
- HR2
- Rv ulramer

ii)

\[ \text{5'UTR} \quad \text{GOI} \quad \text{3'UTR} \quad \text{gDNA} \]

iii)

\[ \text{5'UTR} \quad \text{GOI} \quad \text{3'UTR} \]

- Gene-specific sgRNA
- Fw primer
- Rv primer
Figure 2
Figure 5
Figure 6
Figure 7
CRISPR/Cas9-mediated endogenous C-terminal tagging of Trypanosoma cruzi genes reveals the acidocalcisome localization of the inositol-1,4,5-trisphosphate receptor
Noelia Lander, Miguel Angel Chiurillo, Melissa Storey, Anibal E Vercesi and Roberto Docampo

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