Functional analysis and importance for host cell infection of the Ca$^{2+}$-conducting subunits of the mitochondrial calcium uniporter of Trypanosoma cruzi

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ABSTRACT We report here that Trypanosoma cruzi, the etiologic agent of Chagas disease, possesses two unique paralogues of the mitochondrial calcium uniporter complex TcMCU subunit that we named TcMCUc and TcMCUd. The predicted structure of the proteins indicates that, as predicted for the TcMCU and TcMCUb paralogues, they are composed of two helical membrane-spanning domains and contain a WDXEPXXY motif. Overexpression of each gene led to a significant increase in mitochondrial Ca$^{2+}$ uptake, while knockout (KO) of either TcMCUc or TcMCUd led to a loss of mitochondrial Ca$^{2+}$ uptake, without affecting the mitochondrial membrane potential. TcMCUc-KO and TcMCUb-KO epimastigotes exhibited reduced growth rate in low-glucose medium and alterations in their respiratory rate, citrate synthase activity, and AMP/ATP ratio, while trypomastigotes had reduced ability to efficiently infect host cells and replicate intracellularly as amastigotes. By gene complementation of KO cell lines or by a newly developed CRISPR/Cas9-mediated knock-in approach, we also studied the importance of critical amino acid residues of the four paralogues on mitochondrial Ca$^{2+}$ uptake. In conclusion, the results predict a hetero-oligomeric structure for the T. cruzi MCU complex, with structural and functional differences, as compared with those in the mammalian complex.

INTRODUCTION Trypanosoma cruzi is the etiologic agent of Chagas disease or American trypanosomiasis. No vaccines are available against this vector-borne disease, and several challenges remain in the current anti-parasitic drug treatment including partial or lack of activity in the acute or chronic stage of the disease, respectively, and unwanted side effects (Urbina and Docampo, 2003). New therapeutic approaches with novel modes of action are needed against this and other trypanosomiasis.

One of the most promising sources for novel targets is the trypanosome mitochondrion, which is essential for parasite growth and pathogenesis (Menna-Barreto and de Castro, 2014). Several antiparasitic agents target the mitochondria (Vaidya, 2004; Sen and Majumder, 2008; Monzote and Gille, 2010), but these organelles are largely unexplored targets for trypanosomes (Lisvane Silva et al., 2011). One of the mitochondrial pathways that could provide specific targets is the mitochondrial Ca$^{2+}$ transport mechanism, which is functional in the different life cycle stages of T. cruzi and is important for their infectivity (Chiurillo et al., 2017; Lander et al., 2018).
animal cells this pathway is important for the regulation of mitochondrial metabolism (McCormack et al., 1990), cytoplasmic Ca\(^{2+}\) signaling (Carafoli, 2010), and cell death (Kroemer et al., 2007).

*T. cruzi* has a complex life cycle including replicative and nonreplicative stages in the insect vector (epimastigotes and metacyclic trypomastigotes, respectively) and in its mammalian host (intracellular amastigotes, and cell-derived trypomastigotes, respectively). Although they all possess a functional mitochondrial calcium uniporter (MCU) complex, its role in the regulation of mitochondrial metabolism seems more important in the infective stages (Lander et al., 2018a). Infective stages depend more heavily on an aerobic metabolism of fatty acids and amino acids rather than on glycolysis, as is the case of epimastigotes during the exponential growth phase (Barison et al., 2017; Lander et al., 2018a).

Our finding of the activity of a MCU in *T. cruzi* (Docampo and Vercesi, 1989a,b), together with the finding of its absence in yeast (Carafoli and Lehninger, 1971), were the key (Docampo and Lukes, 2012) to the discovery, first of the modulator mitochondrial calcium uptake 1 (MICU1) (Percoci et al., 2010) and then of the gene encoding the mammalian pore-forming subunit MCU (Baughman et al., 2011; De Stefani et al., 2011). The MCU complex of animal cells was later shown to include additional components such as the MCU parologue and dominant negative subunit MCUb (Raffaello et al., 2013), the scaffolding subunit MCU regulator 1 (MCUR1) (Malilankaraman et al., 2012; Tomar et al., 2016), the essential mitochondrial regulator (EMRE) (Sancak et al., 2013), and MICU2 and MICU3 (Plovanchik et al., 2013). We have found that the trypanosome MCU complex differs in composition and function from the mammalian complex. Trypanosomes possess MCU, MCUb, MICU1, and MICU2 orthologues but lack MCUR1, EMRE, and MICU3 (Huang et al., 2013; Docampo et al., 2014). In contrast to what was reported in animal cells (Raffaello et al., 2013), MCUb is not a dominant negative or inhibitory subunit but a Ca\(^{2+}\)-conducting protein (Chiurillo et al., 2017; Huang and Docampo, 2018). Trypanosomatids possess, in addition, two extra MCU paralogues that were named MCUc, and MCUd that are also Ca\(^{2+}\)-conducting subunits (Huang and Docampo, 2018). These subunits were proposed to form, together with MCU and MCUb, hetero-hexameric complexes in membranes (Huang and Docampo, 2018), in contrast to the in vitro homo-tetramers of recombinant MCU described in fungi (Baradanar et al., 2018; Fan et al., 2018; Nguyen et al., 2018; Yoo et al., 2018), zebrafish (Baradanar et al., 2018), and to the homo-pentamers described in Caenorhabditis elegans (Oxenoid et al., 2016).

In summary, the MCU complex is essential for maintaining the bioenergetics of the cell and for determining life–death decisions (Cardenas et al., 2010; Huang et al., 2013). The *T. cruzi* MCU complex has lineage-specific structural and functional differences as compared with the animal complex (Chiurillo et al., 2017; Lander et al., 2018a,b). Selective interference on this pathway in trypanosomes is therefore a valid alternative for drug targeting and its mechanistic study could have important implications for general mitochondrial physiology. In this work, we characterized the calcium-conducting roles of TcMCUc and TcMCUd and analyzed the phenotypic changes that occur in different stages of the parasite upon their overexpression, ablation, or mutagenesis.

**RESULTS**

**MCUc and MCUd homologues in *T. cruzi***

Two genes encoding putative MCUc and MCUd proteins were identified in the *T. cruzi* genome database (www.tritrypdb.org), *TcMCUc*: TcCLB5.06177.110 and *TcMCUd*: TcCLB5.11367.330. *TcMCUc* and *TcMCUd* predicted proteins of 235 and 219 amino acids, with an estimated molecular mass of 26.8 and 25.5 kDa, respectively. These newly described MCU complex proteins are conserved only in trypanosomatids and have not been described in other species (Supplemental Figure S1) (Huang and Docampo, 2018). All TcMCU paralogues encode proteins with mitochondrial targeting signals (MitoProt II), two transmembrane domains (TM1 and TM2), comparable molecular mass, and sequence similarities in the pore region of TcMCU. It is possible that these paralogues form a hetero-oligomer with TcMCUc and TcMCUd, constituting part of the channel, as it was proposed for the Trypanosoma brucei orthologues (Huang and Docampo, 2018).

**Mitochondrial localization of TcMCUc and TcMCUd and effects of their overexpression**

To confirm the mitochondrial localization of TcMCUc and TcMCUd, we overexpressed their HA-tagged versions (*TcMCUc*-OE and *TcMCUd*-OE) in T. cruzi epimastigotes. Western blot analyses of epimastigote extracts showed protein bands of 26.9 and 26 kDa, compatible with those of the processed forms (in which the mitochondrial targeting signal has been cleaved) of TcMCUc-3xHA and TcMCUd-3xHA, respectively (Figure 1A). TcMCUc and TcMCUd have a second higher band that could correspond to the unprocessed full-length tagged protein. Mitochondrial localization of both HA-tagged proteins was validated by colocalization with the mitochondrial outer membrane protein voltage-dependent anion channel (VDAC; Figure 1B). *TcMCUc*-OE and *TcMCUd*-OE cells had the same growth rate as control cells transfected with the pTREX-n empty vector (Figure 1C).

To ascertain the capacity of the mitochondria of *TcMCUc*-OE and *TcMCUd*-OE cell lines to take up Ca\(^{2+}\), we monitored changes in fluorescence of Calcium Green-5N in digitonin (DIG)-permeabilized epimastigotes in the presence of succinate as substrate (Figure 1, D and E, H and I). There was a significant increase in mitochondrial Ca\(^{2+}\) uptake by either *TcMCUc*-OE or *TcMCUd*-OE epimastigotes, as compared with that of control cells (Figure 1, D and E, H and I). We did not observe modifications in the mitochondrial membrane potential (\(\Delta\psi_m\)) evaluated by changes in safranine O fluorescence in DIG-permeabilized epimastigotes of *TcMCUc*-OE and *TcMCUd*-OE cells as compared with control cells (Figure 1, F and G, J and K).

**Ability of TcMCUc and TcMCUd knockout (KO) mutants to transport Ca\(^{2+}\)**

To investigate the ability of *TcMCUc* and *TcMCUd* to transport Ca\(^{2+}\), we generated null mutants for these two genes (*TcMCUc-KO and *TcMCUd-KO*) using the CRISPR/Cas9 method that we adapted to T. cruzi (Figure 2, A–H, and Supplemental Figure S2) (Lander et al., 2015, 2018a; Chiurillo et al., 2017; Cruz-Bustos et al., 2018). As described under Materials and Methods, *T. cruzi* epimastigotes were transfected with molecular constructs for the constitutive expression of Cas9 nuclease and single guide RNAs (sgRNAs) to target *TcMCUc* or *TcMCUd* genes (Figure 2, A and E). After selection with blasticidin, we obtained clonal populations from these cell lines by limiting dilution. Using specific sets of primers (Figure 2, B and F; Supplemental Table S1), we confirmed by PCR that both *TcMCUc* and *TcMCUd* genes were ablated and replaced by the DNA donor cassette with the resistance marker at the specific loci (Figure 2, C and G, and Supplemental Figure S2). Southern blot analyses confirmed that *TcMCUc* (Figure 2D) and *TcMCUd* (Figure 2H) were absent in genomic DNA (gDNA) of the KO cell lines.

The ability of mitochondria from DIG-permeabilized TcMCUc-KO and TcMCUd-KO epimastigotes to take up Ca\(^{2+}\) was measured using Calcium green-5N in the presence of 20 \(\mu\)M Ca\(^{2+}\) (Figure 2, I–L). Both *TcMCUc*-KO and *TcMCUd*-KO epimastigotes displayed a...
**FIGURE 1:** Analysis of TcMCUc and TcMCUd overexpressing cells. (A) TcMCUc-3xHA (MCUc-OE) and TcMCUd-3xHA (MCUd-OE) overexpression was confirmed by Western blot analysis using anti-HA monoclonal antibodies. WT cells were used as control cell line. α-Tubulin (Tub) was used as loading control. (B) IFA showed colocalization between the green signal of TcMCUc-3xHA (top panel) or TcMCUd-3xHA (bottom panel) and VDAC (red) in the merged image (yellow). DAPI staining (blue) and DIC images are also shown. Scale bars = 10 μm. (C) Growth of control (EV, epimastigotes transfected with pTREXn empty vector), and TcMCUc (MCUc-OE) and TcMCUd (MCUd-OE) epimastigotes in LIT medium. No significant differences in growth rates were found using one-way ANOVA with multiple comparisons. (D) Representative traces of Ca\textsuperscript{2+} uptake by DIG-permeabilized TcMCUc-OE (MCUc-OE) and control (EV) epimastigotes in relative fluorescence units (RFU). The reaction was started after adding 50 μM DIG in the presence of 20 μM free Ca\textsuperscript{2+}, 5 mM succinate and 0.5 μM Calcium Green-5N probe in reaction buffer. (E) Quantification of data from three experiments as shown in D. (F) Changes in mitochondrial membrane potential (ΔΨm) of DIG-permeabilized epimastigotes as detected by changes in safranine O fluorescence in EV, control cells, and MCUc-OE, TcMCUc-OE cells. Cells (5 x 10\textsuperscript{7}) were added to the reaction buffer (2 ml) containing 0.2% BSA, 5 mM succinate, 50 μM EGTA, and 5 μM safranine O. The reaction was started with 50 μM DIG, and 250 μM ADP, 1.5 μM CAT, and 4 μM FCCP were added where indicated. (G) Changes in safranine O fluorescence after addition of ADP to cells used as in F. (H) Representative traces of Ca\textsuperscript{2+} uptake by DIG-permeabilized TcMCUd-OE (MCUd-OE) and control (EV) epimastigotes in RFU. The reaction was started after adding 50 μM DIG using the same experimental conditions and additions as D. (I) Quantification of data from three experiments as shown in H. (J) Changes in ΔΨm of DIG-permeabilized epimastigotes as detected by changes in safranine O fluorescence in EV, control cells, and MCUd-OE, TcMCUd-OE cells. The reaction was started after adding 50 μM DIG using the same experimental conditions and additions as in F. (K) Changes in safranine O fluorescence after addition of ADP to cells as used in J. In E, G, I, and K, values are means ± SD, n = 3; ns, no significant differences; ***P < 0.001 (Student’s t test).
negligible capacity for mitochondrial Ca\(^{2+}\) uptake (Figure 2, I–L). Similarly to TcMCUc-KO, in situ mutated TcMCU\(^{E162Q}\) (see below) abolished the mitochondrial Ca\(^{2+}\) uptake ability in permeabilized epimastigotes (Figure 2, I and J). Ca\(^{2+}\) was taken up by mitochondria of control (scrambled) cells, but not by epimastigotes in which the corresponding gene was ablated. Subsequent dissipation of \(\Delta\psi_m\) by carbonyl cyanide p-trifluoromethoxyphenylhydrazine (FCCP) caused a rapid increase in fluorescence, indicating mitochondrial Ca\(^{2+}\) release (Figure 2, I and K).

To rule out that the defect in mitochondrial Ca\(^{2+}\) uptake in the null mutant cells was due to mitochondrial membrane depolarization, we evaluated the mitochondrial membrane potential (\(\Delta\psi_m\)) of DIG-permeabilized epimastigotes in the presence of succinate as mitochondrial substrate and using safranine O. Knockout of either TcMCUc or TcMCUd did not affect the \(\Delta\psi_m\) (Figure 3, A–D). In conclusion, ablation of TcMCUc or TcMCUd results in a diminished ability of mitochondria to take up Ca\(^{2+}\) without affecting their \(\Delta\psi_m\).

Phenotypic changes in TcMCUc and TcMCUd null mutants

TcMCUc-KO, TcMCU\(^{E162Q}\) (see below), and TcMCUd-KO epimastigotes exhibited similar growth rates in a rich medium liver infusion tryptose (LIT) (Figure 4, A and B). However, the number of cells was significantly reduced in the stationary phase when TcMCUc-KO and TcMCUd-KO epimastigotes were grown in low-glucose LIT medium (Figure 4C), suggesting that under these growth conditions, a marked decrease in mitochondrial Ca\(^{2+}\) uptake affected the bioenergetic functions of this organelle.

To induce the differentiation of TcMCUc-KO and TcMCUd-KO epimastigotes into infective metacyclic trypomastigotes (metacyclogenesis), we incubated these cells in TAU medium as described under Materials and Methods. These cells were able to differentiate to metacyclic trypomastigotes without difference as compared with control cells (transfected with a scrambled sgRNA) (Figure 4D). However, the ability of trypomastigotes to infect tissue-cultured cells (Figure 4B), as well as the replication of intracellular amastigotes (Figure 4F), were significantly impaired in these parasites.

We also evaluated oxygen consumption rates (OCR) under ADP-stimulated (state 3), oligomycin-inhibited (state 4), and FCCP-stimulated (state 3u, uncoupled) conditions in control (scrambled), TcMCUc-KO, and TcMCUd-KO DIG-permeabilized cells in the presence of succinate as respiratory substrate. Control, TcMCUc-KO and TcMCUd-KO mitochondria showed well-coupled respiration, although OCR in the presence of ADP, oligomycin, and FCCP were significantly lower in TcMCUc-KO and TcMCUd-KO mitochondria (Figure 5A). The lower OCR of TcMCUc-KO and TcMCUd-KO mitochondria correlated with lower citrate synthase activity (Figure 5B), suggesting a mitochondrial defect in these cells.

We analyzed the effect of TcMCUc and TcMCUd ablation on cell bioenergetics by measuring the adenosine nucleotide levels of KO and control parasites under normal culture (incubation in LIT medium) and starvation conditions (incubation in phosphate-buffered saline [PBS]), as described previously (Chiruillo et al., 2017). The results indicate that AMP/ATP ratio is significantly increased under starvation conditions in TcMCUc-KO and TcMCUd-KO parasites, as compared with control epimastigotes (Figure 5C). However, this result was not correlated with the number of autophagosomes per cell observed in KO parasites under starvation (PBS), as detected by immunofluorescence microscopy using antibodies against ATG8.1 (Figure 5D). ATG8.1 is a marker of autophagosomes that has been used previously to evaluate autophagy in T. cruzi (Alvarez et al., 2008). We did not observe differences in the percentage of autophagosomes per cell between TcMCUc-KO or TcMCUd-KO and control cells.

Microscopy counts of cell viability after treatment with H\(_2\)O\(_2\) or C2-ceramide showed that TcMCUc-KO and TcMCUd-KO epimastigotes exhibited a significant increased ability to resist oxidative stress (225 µM H\(_2\)O\(_2\)) (Figure 5E) and the effect of proapoptotic agents, such as C2-ceramide (Figure 5F).

Analysis of conserved amino acid residues in the selectivity filter of predicted MCU paralogues

Recent Cryo-EM studies of the recombinant MCU from fungi (Baradaran et al., 2018; Fan et al., 2018; Nguyen et al., 2018; Yoo et al., 2018) and zebrafish (Baradaran et al., 2018) have revealed the formation of homo-tetramers where each of the monomers contribute to the formation of two rings of acidic residues in the selectivity filter. One ring is of larger diameter with each subunit contributing with an aspartate (D), and the second is narrow with each subunit contributing a glutamate (E), analogous to the high-affinity Ca\(^{2+}\) site formed by the EEEE locus of Ca\(^{2+}\) channels (Nguyen et al., 2018). Taking into account that the MCU channel in trypanosomes is a hetero-oligomer and that each subunit is essential for mitochondrial Ca\(^{2+}\) uptake, we aimed at studying whether substitution of critical D and E residues in the WDXE PXXY domain of each subunit could affect mitochondrial Ca\(^{2+}\) uptake. We included in this analysis a D residue which is located N-terminal to the WDXE PXXY domain and is conserved in three (MCU, MCUC, and MCUD) of the MCU paralogues but not in human MCU (Supplemental Figure S3).

Mitochondrial Ca\(^{2+}\) transport is impaired in permeabilized TcMCUc epimastigotes. However, we were able to reconstitute Ca\(^{2+}\) transport in these cells by expressing an exogenous copy of TcMCU (Chiruillo et al., 2017) (Figure 6A). We also introduced mutations in the exogenous gene in highly conserved residues to evaluate their ability to restore Ca\(^{2+}\) transport in TcMCU-KO cells. Western blot analysis confirmed the expression of these proteins (Figure 6A). Complementation with TcMCU\(^{E227N}\) or TcMCU\(^{D226H}\) mutants, but not with the TcMCU\(^{E227Q}\) mutant, was able to partially restore mitochondrial Ca\(^{2+}\) uptake (Figure 6A) and the effect of proapoptotic agents, such as C2-ceramide (Figure 5F). The results suggest that the glutamate 226 (E226) at the narrow selectivity filter of TcMCU is essential for Ca\(^{2+}\) transport, whereas D223 and D221 are important but not essential.

TcMCUb-KO cells exhibit a significantly reduced growth rate (Chiruillo et al., 2017), and all attempts to restore mitochondrial Ca\(^{2+}\) uptake by gene complementation of TcMCUB-KO cells were unsuccessful. Therefore, we generated in situ TcMCUB-WDXE PXXY mutants by using a CRISPR/Cas9 strategy (Supplemental Figures S4, A and B, and S5, A and B) to introduce mutations in D (D161) and E (E164) residues of this motif (Supplemental Figure S3). Western blot analysis confirmed the expression of these mutated proteins (Figure 6D). In situ mutations in TcMCUB\(^{D161N}\) resulted in a significant reduction of Ca\(^{2+}\) uptake, whereas the TcMCUB\(^{E164Q}\) substitution abolished the mitochondrial ability to take up Ca\(^{2+}\), similarly to TcMCUc-KO epimastigotes (Figure 6, E and F).

TcMCUc and TcMCUd share the same amino acid sequence for the selectivity filter: WNLVEPMTY. Both proteins lack the D residue of WDXE PXXY motif and, like TcMCU, show an aspartate residue before it (Supplemental Figure S3). We then investigated whether introducing mutations in D and E residues of the putative DWNLVE-PMTY selectivity filter of exogenous TcMCUc and TcMCUd genes could affect their ability to rescue mitochondrial Ca\(^{2+}\) uptake in TcMCUc-KO and TcMCUd-KO cells. We also introduced a mutation in E162 residue of TcMCUc by the same knock-in strategy as that used for TcMCUb (Supplemental Figure S5, C and E). Western blot
**FIGURE 2**: Ca\(^{2+}\) uptake by TcMCUc and TcMCUd KOs. (A) Schematic representation of the strategy used to generate a TcMCUc-KO mutant by CRISPR/Cas9-induced homologous recombination. A double-stranded gDNA break was produced by Cas9 at nt +468 of the TcMCUc ORF (708 base pairs). DNA was repaired with a Bsd cassette containing 100–base pair homologous regions spanning from nt -44 to +56 and from nt +677 to +776 of the TcMCUc locus. (B) Primers (arrows) that were used to verify gene disruption by PCR. The intact locus generates a PCR product of 923 base pairs, while the disrupted locus generates a fragment of 701 base pairs. (C) TcMCUc was disrupted at its genomic locus in the KO cell line. Lanes: 1, 1-kb plus ladder; 2, WT; 3, TcMCUc-KO mutant cell line; 4, PCR negative control. (D) Southern blot analysis of WT and TcMCUc-KO (KO) epimastigotes. The blot was hybridized with a 409–base pair
analyses confirmed the expression of these proteins in transfected epimastigotes (Figure 7, A and D). Complementation with a TcMCUcD157N or TcMCUdE146Q mutant, but not with the TcMCUcE162Q or TcMCUdE144Q mutant, was able to restore mitochondrial Ca\textsuperscript{2+} transport (Figure 7, B and C, E and F, respectively). As shown above, mitochondria of TcMCUcD157N epimastigotes obtained by in situ mutation were also unable to take up Ca\textsuperscript{2+} (Figure 2, I and J). As occurs with TcMCU and TcMCUb described above, the glutamate residues E162 of TcMCUc and E146 of TcMCUd are essential, whereas the aspartates D157 of TcMCUc and D141 of TcMCUd are important but not essential for Ca\textsuperscript{2+} transport. Interestingly, while in the overexpression experiments, the unprocessed TcMCUc (top band Figure 1A) is weaker, it is the opposite in the complemented TcMCUc-KO (Figure 7A). For TcMCUd, the two bands have equivalent intensity in the overexpression experiment (Figure 1A) but the bottom band is relatively more abundant in the complemented cells (Figure 7D). This could suggest that either their processing is different according to the expression level or the differences are due to competition with wild-type (WT) proteins in the overexpression experiment.

In previous work, we reported that human MCU was unable to complement TcMCU-KO epimastigotes (Chirillo et al., 2017). However, it has been reported that human MCU alone is not sufficient to reconstitute Ca\textsuperscript{2+} transport in yeast unless it is expressed together with EMRE (Kovacs-Bogdan et al., 2014). Therefore, we attempted to coexpress HsEMRE with HsMCU in TcMCU-KO epimastigotes. Both proteins colocalized to:

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**FIGURE 3:** Mitochondrial membrane potential in TcMCUc and TcMCUd KOs. (A) Changes in mitochondrial membrane potential (ΔΨ\textsubscript{m}) of DIG-permeabilized epimastigotes as detected by changes in safranine O fluorescence in cells transfected with scrambled sgRNA, Scr, and MCUc-KO, TcMCUc-KO cells. The reaction was started with 50 μM DIG, and 250 μM ADP, 1.5 μM CAT, and 4 μM FCCP were added where indicated. (B) Changes in safranine fluorescence after addition of ADP of cells as used in A. (C) Changes in ΔΨ\textsubscript{m} of DIG-permeabilized epimastigotes as detected by changes in safranine O fluorescence in cells transfected with scrambled sgRNA (Scr), and MCUd-KO, TcMCUd-KO cells. The reaction was started after adding 50 μM DIG using the same experimental conditions and additions as in A. (D) Changes in safranine fluorescence after addition of ADP of cells as used in C. In B and D, values are mean ± SD, n = 3, ns, no significant differences (Student’s t test).

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biotin-labeled fragment of TcMCUc (nt +240 to +649). Arrow indicates the expected restriction band recognized by the probe. (E) Schematic representation of the strategy used to generate a TcMCUd-KO mutant by CRISPR/Cas9-induced homologous recombination. A double-stranded gDNA break was produced by Cas9 at nt +252 of the TcMCUd ORF (680 base pairs). DNA was repaired with a Bsd cassette containing 100–base pair homologous regions spanning from nt −100 to +1 and from nt +630 to +730 of the TcMCUd locus. (F) Primers (arrows) used to verify gene replacement by PCR. The intact locus generates a PCR product of 899 base pairs, while the disrupted locus generates a fragment of 638 base pairs. (G) TcMCUd was disrupted at its genomic locus by the Bsd gene in the KO cell line. Lanes: 1, 1-kb plus ladder; 2, WT; 3, TcMCUd-KO mutant cell line; 4, PCR negative control. (H) Southern blot analysis of WT and TcMCUd-KO (KO) epimastigotes. The blot was hybridized with a biotin-labeled probe corresponding to 435 base pairs of TcMCUd (nt +1 to +435). Arrow indicates the expected restriction band recognized by the probe. (I) Ca\textsuperscript{2+} uptake by DIG-permeabilized epimastigotes in RFU. Scr, cells transfected with scrambled sgRNA in the absence of presence of 5 μM ruthenium red (+ RR); MCUc-KO, TcMCUc-KO cells; MCUc-E162Q, TcMCUc-E162Q cells. The reaction was started after adding 50 μM DIG in the presence of 20 μM CaCl\textsubscript{2}. Where indicated, 4 μM FCCP was added. (J) Quantification of data from three experiments as shown in I. Relative Ca\textsuperscript{2+} uptake at 600 s compared with that of epimastigotes transfected with scrambled sgRNA. Values in J and L are mean ± SD (n = 3); ***P < 0.001; one-way ANOVA.
the mitochondrion of T. cruzi, as determined by immunofluorescence analysis (IFA) (Supplemental Figure S6A), and Western blot analyses confirmed the expression of the proteins (Supplemental Figure S6, B C). However, they were unable to reconstitute mitochondrial Ca\textsuperscript{2+} transport in permeabilized epimastigotes (Supplemental Figure S6D). Supplemental Figure S7 shows the complete Western blots.

**DISCUSSION**

Our studies revealed that TcMCUc and TcMCUd KO epimastigotes are viable, replicate normally in rich medium, and are able to differentiate into trypomastigotes. However, these mutant cells show a lower growth rate in low-glucose LIT medium, lower rates of respiratory activity, and significantly reduced infectivity and replication inside host cells. In addition, TcMCUc and TcMCUd KO epimastigotes evidenced an increased capacity to survive an oxidative stress caused by H\textsubscript{2}O\textsubscript{2} or a pro-apoptotic stress caused by C2-ceramide treatment. TcMCUc and TcMCUd, as occurs with TcMCU and TcMCUb (Chiurillo et al., 2017), are essential for mitochondrial Ca\textsuperscript{2+} uptake. Conserved Glu (E) residues in the WDXXEPXXY motif of MCU, MCUb, MCUc and MC Ud are critical, while conserved Asp (D) residues are important for Ca\textsuperscript{2+} uptake. The fact that individual ablation of genes encoding each one of the MCU subunits or substitution of critical residues in WDXXEPXXY motif causes suppression of mitochondrial Ca\textsuperscript{2+} uptake suggests that all of them are essential in a putative hetero-oligomeric structure of the T. cruzi MCU complex. Each subunit may form part of the oligomer in the same way that several MCU subunits interact in animal and fungal cell mitochondria to form the MCU complex.

The deficient ability of TcMCUc and TcMCUd KO epimastigotes to grow in low-glucose medium, and of trypomastigotes to infect and to replicate inside host cells as amastigotes, is consistent with their deficient respiratory activity, the more aerobic metabolism of epimastigotes during the stationary phase of growth (Barison et al., 2017), and the relevance on mitochondrial metabolism for the active invasion of host cells by trypomastigotes (Schenkman et al., 1991) and for replication of intracellular amastigotes (Demoulin and Burleigh, 2018), respectively. As occurs with TcMCU-KO epimastigotes, TcMCUc and TcMCUd KO epimastigotes grow slowly in low-glucose LIT medium, but in contrast to them they have lower respiratory rate suggesting that, as we have found for TcMCUb (Chiurillo et al., 2017), other potential functions of these subunits independent of Ca\textsuperscript{2+} transport are important. It would be interesting to know whether these functions are related to their susceptibility to stress conditions.

Structural studies of the animal (Baradaran et al., 2018) and fungi (Baradaran et al., 2018; Fan et al., 2018; Nguyen et al., 2018; Yoo et al., 2018) recombinant MCU subunit have described the formation of homo-tetramers. The selectivity filter is formed by symmetrical arrangement of WDXXEPXXY sequences in TM2 from each monomer around the pore. The D residues form an acidic mouth with a radius of \(\sim 2.2\) Å (Site 1), while the E residues form a second acidic ring (Site 2).
In contrast, mutations of the E residues of each monomer completely ablated mitochondrial Ca\(^{2+}\) transport. Moreover, in a previous work we were able to restore mitochondrial Ca\(^{2+}\) uptake in TcMCU-KO cells when they were transfected with TcMCU(D219W/D219V) mutant, indicating that these two residues are not important for Ca\(^{2+}\) transport in T. cruzi (Chiurillo et al., 2017). The residues corresponding to these two residues in other organisms are located in TM1 and the loop region near the WDXRXPYX motif but outside the channel entrance (Oxenoid et al., 2016; Baradaran et al., 2018; Fan et al., 2018; Nguyen et al., 2018; Yoo et al., 2018). However, the fact that substitutions in the conserved D residues in TcMCU (D221), TcMCUc (D157), and TcMCUd (D141) reduced mitochondrial Ca\(^{2+}\) uptake suggests that these D residues located near the WDXRXPYX motif are important for the function, or even could be part of the selectivity filter of T. cruzi MCU.

Our previous work in the related trypanosomatid T. brucei (Huang and Docampo, 2018) demonstrated that each of the four MCU monomer subunits interact with each other, as revealed by 1) the formation of large protein complexes detected by blue native gel separation of mitochondrial proteins and labeling with antibodies against all the subunits; and 2) coimmunoprecipitation of the subunits. The strength of their interactions, as evaluated by membrane-based yeast two hybrid assays, suggested the formation of hetero-hexamers (Huang and Docampo, 2018). Deletion of each monomer would affect the formation of the complex and result in loss of mitochondrial Ca\(^{2+}\) transport.

Interestingly, the recombinant pore domains of MCU from Caenorhabditis elegans (Oxenoid et al., 2016) form homo-pentamers while those from several fungi, like Neurospora crassa (Yoo et al., 2018), Neosartorya fischeri (Nguyen et al., 2018), Metarhizium acridum (Fan et al., 2018), Fusarium graminearum (Fan et al., 2018), and Cypellophora europea (Baradaran et al., 2018) and those from zebrafish (Baradaran et al., 2018) form homo-tetramers in vitro. However, which is the oligomeric state in vivo and how animal and fungal MCU interacts with its membrane partners, like MCUB and others in those species, remain to be investigated.

Our attempts to complement TcMCU-KO epimastigotes by coexpressing HsMCU and HsEMRE failed despite their colocalization to the mitochondrion. However, it is possible that the proteins did not insert properly in the inner membrane or with the right topology, failed to interact, or did not form part of the MCU complex.

with a pore radius of less than 1 Å (Baradaran et al., 2018). This Site 2 represents the high-affinity binding site for Ca\(^{2+}\) in MCU. Accordingly, mutations of the D residues located in the filter region of TcMCU (D223) or TcMUCb (D161), or the D residues located N-terminal to the selectivity filter of TcMCU (D221), TcMUCc (D157), or TcMCUd (D141), reduced but did not completely abolish mitochondrial Ca\(^{2+}\) transport.
Interestingly, all *T. cruzi* MCU complex monomers lack the serine amino acid (Ser259 in the human MCU) that has been proposed to be responsible for the sensitivity of the uniporter to the ruthenium red derivative Ru360 (Baughman et al., 2011). In contrast with these structural differences, mitochondrial Ca\(^{2+}\) transport in *T. cruzi* is sensitive to Ru360 (unpublished results), suggesting that there is another target for Ru360 besides Ser259.

In conclusion, we demonstrated the Ca\(^{2+}\)-conducting activity of all subunits of the *T. cruzi* MCU complex and the involvement of conserved D and E residues in the uniporter function. The two novel subunits present only in trypanosomatids (TcMCUc and TcMCUb), together with the TcMCUb subunit, have Ca\(^{2+}\) transport-independent roles and are important for epimastigote growth in low-glucose medium, for trypanotogastode infection of host cells, and for intracellular amastigote replication. In addition, we report for the first time a novel CRISPR/Cas9 strategy for doing gene knock-ins in *T. cruzi*.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Platinum Taq DNA Polymerase High Fidelity, Calcium Green-5N, MitoTracker Deep Red FM, Alexa–conjugated secondary antibodies, ATP determination kit, Pierce ECL Western blotting substrate, BCA Protein Assay Kit, North2South Biotin Random Prime Labeling Kit, North2South Chemiluminescent Hybridization and Detection Kit, and HA epitope tag monoclonal antibody, and hygromycin B were from Thermo Fisher Scientific. Blasticidin S HCI, BenchMark prestained protein ladder, BenchMark protein ladder,
Alexa Fluor–conjugated secondary antibodies, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Life Technologies. Benzonase Nuclease was from Novagen (EMD Millipore, Billerica, MA). Wizard Plus SV Miniprep Purification System, Wizard SV Gel and PCR Clean-Up System, GoTaq DNA Polymerase, 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 mAb (clone 3F10) was purchased from Roche Applied Science. The pMOTag23M vector (Oberholzer et al., 2006) was from Thomas Seebeck (University of Bern, Bern, Switzerland). DNA oligonucleotides were purchased from Exxtend Biotecnologia (Campinas, Brazil). Precision Plus Protein Dual Color Standards and nitrocellulose membranes were from Bio-Rad. Anti-c-Myc mAb (clone 9E10) was from Santa Cruz Biotechnology (Dallas, TX). Carboxyatractyloside (CAT), oligomycin, safranine O, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), Benzonase Nuclease, anti-tubulin mAb, anti-Flag antibody, puromycin, G418, N-acetyl-d-sphingosine, mammalian cell protease inhibitor mixture (Sigma P8340), other protease inhibitors, and all other reagents of analytical grade were from Sigma (St. Louis, MO). Rabbit polyclonal antibody against TbVDAC was a gift from Minu Chaudhuri (Meharry Medical College, Nashville, TN). Rabbit polyclonal antibody against TcATG8.1 was a gift from Vanina Alvarez (Universidad Nacional de San Martin, Argentina).

**Cell culture**

*T. cruzi* Y strain epimastigotes were cultured in LIT medium containing 10% heat-inactivated fetal bovine serum (FBS) at 28°C.
(Bone and Steinhart, 1956). CRISPR/Cas9 mutant cell lines were maintained in medium containing 250 μg/ml G418 and 10 μg/ml blasticidin or 5 μg/ml puromycin. When KO cell lines were transfected with constructs made in pTREX-h for complementation experiments, hygromycin B (250 μg/ml) was added to the culture medium. Epimastigotes overexpressing TcMCUc-3xHA or TcMCUd-3xHA were cultured in medium containing 250 g/ml G418. The growth rate of epimastigotes was determined by counting cells in a Muse Cell Analyzer. Tissue culture cell-derived trypanosomatids were obtained from Vero cells infected with metacyclic trypanosomatids obtained as described below. T. cruzi trypanosomatids were collected from the culture medium of infected host cells using a modification of the method of Schmatz and Murray (1982) as described previously (Moreno et al., 1994). Vero cells were grown in RPMI supplemented with 10% FBS and maintained at 37°C with 5% CO₂.

**TcMCUc and TcMCUd overexpression**

TcMCUc and TcMCUd open reading frames (ORF) (708 and 660 nt, respectively) were PCR amplified using T. cruzi Y strain gDNA as template (primers 1, 2, 10, and 11; Supplemental Table S1) and cloned into the pTREX-n/3xHA vector (Chiurillo et al., 2017) by restriction sites XbaI/Xhol. Gene cloning was confirmed by PCR and sequencing, and constructs were subsequently used to transfect T. cruzi epimastigotes. TcMCUc and TcMCUd overexpression was confirmed by Western blot analysis using anti-HA antibodies.

**Knockout of TcMCUc and TcMCUd**

Selection of protospacers was performed using EuPaGDT (Eukaryotic Pathogen CRISPR guide RNA Design Tool, http://grna.ctegd.ua.gov/). The sgRNA sequences to target the TcMCUc and TcMCUd genes (TryTripDB identifier TcCLB.506181.97 and TcCLB.511367.330, respectively) were amplified by PCR from plasmid pUC_sgRNA using a specific forward primer including the protospacer sequence and a common reverse primer (primers 3, 4, and 12; Supplemental Table S1), as described previously (Lander et al., 2015). PCR-amplified fragments were cloned into Cas9/ pTREX-n by the BamHI restriction site (Lander et al., 2015) to generate TcMCUc-sgRNA/Cas9/pTREX-n and TcMCUd-sgRNA/ Cas9/pTREX-n constructs. The sgRNA orientation was verified by PCR using the specific sgRNA forward primers and the HX1 reverse primer (primers 3, 5, and 12; Supplemental Table S1) (Lander et al., 2015) and also by sequencing. A scrambled sgRNA cloned in Cas9/pTREX-n was used as control. DNA donor cassettes designed to promote homologous directed-repair (HDR) and disruption of TcMCUc or replacement of TcMCUd ORFs were obtained by PCR using a set of 120 nt primers (ultramers). Each one of the ultramers had 20 nt annealing on the Blasticidin-S deaminase (Bsd) gene (primers 6, 7, 13, and 14; Supplemental Table S1). Plasmid constructs TcMCUc-sgRNA/Cas9/pTREX-n or TcMCUd-sgRNA/ Cas9/pTREX-n and linear Bsd cassette were used to cotransfect T. cruzi epimastigotes. After selection with 250 μg/ml G418 and 10 μg/ml blasticidin, TcMCUc gene disruption and TcMCUd replacement were verified by PCR using primers 8, 9, 15, and 16 (Supplemental Table S1).

**Molecular constructs to complement TcMCU-KO, TcMCUc-KO, and TcMCUd-KO mutants**

We generated molecular constructs to revert null-mutant phenotypes of TcMCU, TcMCUc, and TcMCUd as well as to investigate critical amino acid residues in the selectivity filter region of the three proteins. We used TcMCU-KO epimastigotes that were characterized in a previous work (Chiurillo et al., 2017) and the TcMCUc-KO and TdMCUd-KO cells obtained in this work. To make the complementation constructs, we followed a two-step-PCR strategy for TcMCUc we used primers that simultaneously eliminate the protospacer adjacent motif (PAM) sequence specific for the sgRNA used to obtain the TcMCUc-KO cells and mutate critical residues (Supplemental Table S1; primers 17–20), whereas for TcMCUd we first mutated the PAM sequence and then this TcMCUd-PAM sequence was used as a template to introduce the desired mutations in the selectivity filter DNA sequence (Supplemental Table S1; primers 21–26). In the case of TcMCU, we used the pTREX-h-TcMCU-PAM-PAH plasmid as a template (Chiurillo et al., 2017) to introduce mutations by overlap extension PCRs (Supplemental Table S1; primers 27–32). By eliminating the PAM sequence the constitutively expressed Cas9 is not able to target the inserted sequence. We also included a C-terminal 3x-c-Myc tag in order to detect the overexpressed protein using the anti-c-Myc antibody. The molecular constructs made were TcMCU221N, TcMCU223N, and TcMCU226Q for TcMCU; TcMCU2157N and TcMCU2162Q for TcMCUc; and TcMCUc2141N and TcMCUc2144Q for TcMCUd (Supplemental Figure S3). All constructs were made in pTREX-h-3xc-Myc vector (Chiurillo et al., 2017), which confers hygromycin resistance using XbaI/Xhol restriction sites.

**CRISPR/Cas9-induced knock-in mutations in TcMCUb and TcMCUc genes**

To achieve knock-in mutations in critical amino acid residues in the selectivity filter region of TcMCUb and TcMCUc, we adapted the method developed for CRISPR/Cas9-mediated endogenous C-terminal tagging in T. cruzi (Lander et al., 2016). Therefore, we provided donor DNA molecules that by inducing HDR introduce mutations in the genome of parasites and also tag the mutated genes to follow their expression by Western blot and IFA (Supplemental Figure S4). Specific sgRNA targeting the region coding for the selectivity filter of TcMCUb was designed and obtained following methodology described above (primer 33; Supplemental Table S1), which in the case of TcMCUb was the same designed to disrupt it. To generate donor DNA constructs, we performed a two-step-PCR strategy in which we amplified, in two separate PCRs, a 3' portion of the gene including a mutated PAM sequence for the specific sgRNA used for the knock-in strategy (primers 34, 35, 42, and 43; Supplemental Table S1), while in the second reaction the 3xc-Myc tag sequence and the puromycin resistance gene were amplified using the pMOTag23M vector as template (primers 36, 37, 44, and 45; Supplemental Table S1) (Oberholzer et al., 2006). Subsequently, we used two 120 nt ultramers in an overlap extension PCR including the two above-mentioned fragments as templates (primers 37, 38, 39, 45, and 46; Supplemental Table S1). Specific forward ultramers including selectivity filters mutations were used to obtain donor DNAs to generate these mutant cells: TcMCUb2161N and TcMCUb2164Q for TcMCUb and TcMCUc2164Q for TcMCUc. For TcMCUb, a forward ultramer with WT sequence was also used to obtain TcMCUbcontrol construct. Epimastigotes cotransfected with sgRNA/Cas9/pTREX-n and DNA donor were cultured for 5 wk with G418 and puromycin for selection of double-resistant parasites. Endogenous gene tagging was verified by PCR from gDNA using specific primer sets (primers 9, 40, 41, and 47; Supplemental Table S1) and by Western blot analysis.

**Cell transfections**

T. cruzi Y strain epimastigotes were transfected as described previously (Chiurillo et al., 2017). Briefly, T. cruzi epimastigotes in early
Exponential phase (4 × 10^7 cells) were washed with PBS, pH 7.4, at room temperature (RT) and transfected in ice-cold Cytomix (120 mM KCl, 0.15 mM CaCl_2, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EDTA, 5 mM MgCl_2, pH 7.6) containing 25 μg of each plasmid construct and 25 μg of donor DNA in 4-μm electroporation cuvettes with three pulses (1500 V, 25 μF) delivered by a Gene Pulser Xcell Electroporation System (Bio-Rad). Transfected epimastigotes were cultured in LIT medium supplemented with 20% heat-inactivated FBS until stable cell lines were obtained. When needed, the antibiotic concentration used for drug selection and maintenance was 250 μg/ml G418, 10 μg/ml blasticidin, 5 μg/ml puromycin, or 250 μg/ml hygromycin. Parasite clones were obtained by limiting dilution.

**Southern blot analysis**

To confirm gene KO's, 25 μg of gDNA from control (transfected with scramble sgRNA), TcMCUc-KO, or TcMCUd epimastigotes was digested with PstI enzyme, resolved on a 0.8% agarose gel and restriction fragments were transferred to nylon membranes. PCR-amplified fragments corresponding to 409 and 435 nt of TcMCUc and TcMCUd, respectively (primers 10, 48, 49, and 50; Supplemental Table S1), were labeled with biotin by random primer method using the NorthSouth2 Biotin Random Prime Labeling Kit. Hybridization, posthybridization washes, and detection were done with the NorthSouth Chemiluminescent Hybridization and Detection Kit, following manufacturer's recommendations. Signal detection was done using an UVIteq Alliance Gel Documentation System (UVIteq, Cambridge, UK).

**Western blot analysis**

Western blots were carried out as described previously (Lander et al., 2010, 2013). Briefly, parasites were harvested and washed twice in PBS and subsequently resuspended in radio-immunoprecipitation assay buffer (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, 100 M N-(trans-epoxyxysuccinyl)-l-leucine 4-guanidinobutylamide (E64), and Benzonase Nuclease (25 U/ml culture). The cells were then incubated for 1 h on ice, and protein concentration was determined by BCA protein assay. Thirty micrograms of protein from each cell lysate was collected and resuspended in 10% SDS–polyacrylamide gels. Electrophoresed proteins were transferred onto nitrocellulose membranes with a Bio-Rad transblot 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, that is, monoclonal anti-HA (1:5000), 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 immunoglobulin G [IgG] or goat anti-rabbit IgG, HRP-conjugated antibody, diluted 1:10,000). Membranes were washed three times with PBS-T, then blots were incubated with Pierce ECL Plus Substrate, and images were obtained and processed with a C-DiGit Blot Scanner (LI-COR Biosciences).

**IFA**

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% bovine serum albumin (BSA), 1% fish gelatin, 50 mM NH_4Cl, and 5% goat serum overnight at 4°C. Then, cells were incubated with a primary antibody (monoclonal anti-FA-Tag [1:500], monoclonal anti-c-Myc-tag [1:10], or rabbit polyclonal 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 secondary antibody (1:1000). Then, cells were washed and mounted on slides using Fluoromount-G mounting medium containing 5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) to stain DNA. Controls were done as described above but in the absence of a primary antibody. Differential interference contrast (DIC) and fluorescence optical images were captured with a confocal microscope Leica TCS SP5 II, with a 100x objective (1.44 aperture) under non-saturating conditions that uses photomultiplier tubes for detection of emission, and LAS AF software (Leica, Wetzlar, Germany) for acquisition and processing of digital images.

**Metacyclogenesis**

Metacyclic trypomastigotes were obtained following the protocol described by Bourguignon et al. (1998) with minor modifications. Epimastigotes were obtained after 4 d in LIT medium and incubated for 2 h in triatome artificial urine (TAU) medium (190 mM NaCl, 17 mM KCl, 2 mM MgCl_2, 2 mM CaCl_2, 0.035% sodium bicarbonate, 8 mM phosphate, pH 6.9, at RT). Then, parasites were incubated for 96 h in TAU 3AAG medium (TAU medium supplemented with 10 mM L-proline, 50 mM sodium L-glutamate, 2 mM sodium L-aspartate, and 10 mM glucose). To increase the number of metacyclic forms to infect Vero cells, the contents of the flask were collected and resuspended in medium containing fresh FBS and incubated at 37°C for 20 h. The complement in fresh FBS kills epimastigotes, whereas metacyclic trypomastigotes survive. Samples were harvested from the TAU 3AAG plus FBS-containing medium at days 5 and 10 of cultivation.

**In vitro infection assay**

Gamma-irradiated (2000 radiation-absorbed doses) Vero cells (4.5 × 10^5 cells) were plated onto sterile coverslips in a 12-well plate and incubated overnight at 35°C, 7% CO_2, in RPMI medium plus 10% fresh FBS. Tissue culture-derived trypomastigotes were incubated at 4°C overnight to allow amastigotes to settle from swimming trypomastigotes. Trypomastigotes from the supernatants of these collections were counted and used to infect the coverslips at a 10:1 ratio of parasites to host cells. At 4 h postinfection, coverslips were washed extensively with Hank’s balanced salt solution, followed by PBS, pH 7.4, to remove any extracellular parasites. Coverslips were fixed immediately in 4% paraformaldehyde in PBS, pH 7.4, at 4°C for 30 min. Coverslips were washed once with PBS and mounted onto glass slides in Fluoromount-G containing 15 μg/ml DAPI, which stains host and parasite DNA. Coverslips were viewed on an Olympus BX60 microscope to quantitatively count the number of host cells that contained intracellular parasites and the number of intracellular parasites per cell in randomly selected fields. Three hundred host cells were counted per sample in three independent experiments. To quantify amastigote replication, the following modifications were used: host cells were infected at a ratio of 10 parasites to one host cell, and coverslips were allowed to incubate for 48 h postinfection at 35°C, 7% CO_2, prior to fixation and DAPI staining. Coverslips were mounted onto glass slides and analyzed by fluorescence microscopy. Amastigotes in infected cells were counted using a 100x objective.
Cellular respiration
The OCR of DIG-permeabilized epimastigotes was measured using a high-resolution respirometer (Orboros Oxygraph-2k, Innsbruck, Austria) with DatLab 4 software for data acquisition and analysis, and calibrated as reported by its manufacturer. Cells (1 × 10⁷) were incubated at 28°C in a 2 ml reaction volume containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, pH 7.2, 2.5 mM K₂PO₄, 1 mM MgCl₂, 50 μM EGTA, 5 mM succinate, and 25 μM DIG. OCR was calculated as the negative time derivative of oxygen concentration measured in the closed respirometer chambers and expressed per milligram of protein. Data were recorded at 2-s intervals, and 10 data points were used to calculate the slope of the OCR plot through a polynomial fit with DatLab 4 software, as described (Pesta and Gnaiger, 2012).

Citrate synthase activity
Citrate synthase activity was measured using a previously described protocol (Figueira et al., 2011) adapted to trypanosomes (Chiurillo et al., 2017). Briefly, the conversion of oxaloacetate and acetyl-CoA to citrate and SH-CoA was monitored by quantification of the colorimetric product thionitrobenzoic acid (Shepherd and Garland, 1969). T. cruzi epimastigotes in early exponential phase (~1 × 10⁸ cells) were washed twice with PBS and incubated in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, and 25 μM Benzonase Nuclease) for 10 min on ice. Then, proteins were quantified by BCA protein assay and 260-μl reactions were set up in buffer containing 5 μg protein, 250 μM oxaloacetate, 50 μM acetyl-CoA, 100 μM 5,5-dithio-bis(2-nitrobenzoic acid), and 10 mM Tris-HCl, pH 8.0. The increase in absorbance at 412 nm was monitored for 20 min at 28°C using a microplate reader (PowerWave XS 2; BioTek Instruments, Winooski, VT). Values of Vₘₙₐₓ were normalized taking scrambled cell line as reference value.

Mitochondrial membrane potential
Mitochondrial membrane potential was assessed spectrofluorometrically using the indicator dye safranine O, as described previously (Figueira et al., 2012; Chiurillo et al., 2017). Briefly T. cruzi epimastigotes (5 × 10⁷ cells) were incubated at 28°C in reaction buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH buffer, pH 7.2, 1 mM MgCl₂, 2.5 mM potassium phosphate; 1.95 ml) containing 5 mM succinate, 0.2% BSA, 50 μM EGTA, and 5 μM safranine O, and the reaction was started with DIG (50 μM). ADP (250 μM), CAT (1.5 μM), and FCCP (4 μM) were added to the media at different time points. Fluorescence changes were monitored on a Hitachi F-7000 spectrofluorometer (excitation at 495 nm and emission at 586 nm).

Mitochondrial calcium transport
The uptake of Ca²⁺ by permeabilized T. cruzi epimastigotes was assayed by fluorescence of Calcium Green-5N probe at 28°C, as described previously (Chiurillo et al., 2017). Briefly, cells were collected by centrifugation at 1000 × g for 7 min and washed twice with buffer A with glucose (BAG; 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM D-glucose, and 50 mM HEPES, pH 7.0). Epimastigotes were resuspended to a final density of 1 × 10⁶ cells/ml in BAG and kept on ice. Before each experiment, a 50-μl aliquot of T. cruzi epimastigotes (5 × 10⁷ cells) was added to the reaction buffer (125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH buffer, pH 7.2, 1 mM MgCl₂, 2.5 mM potassium phosphate; 1.95 ml) containing 5 mM succinate, 50 μM EGTA, and 0.5 μM Calcium Green-5N. Ca²⁺ needed to reach 20 μM free Ca²⁺ was added according to the calculation made with software Maxchelator Calculator v1.2 (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaEGTA-NIST.htm). Mitochondrial Ca²⁺ uptake was initiated by the addition of 50 μM DIG. FCCP (4 μM) was added in each experiment when Ca²⁺ uptake reached a steady state level. Fluorescence changes were monitored in the Hitachi F-7000 spectrofluorometer (excitation at 506 nm and emission at 532 nm).

Autophagy assay
Evaluation of autophagy was performed by determining expression of the TcAtg8.1 autophagy marker in T. cruzi epimastigotes grown in LIT medium and under starvation conditions (PBS). Autophagosome formation was estimated by immunofluorescence analyses using anti-TcATG8.1 antibody as described (Alvarez et al., 2008). For starvation induction, mid-log parasites were washed twice with PBS, resuspended in the same buffer at a concentration of 5 × 10⁷ cells/ml, and incubated for 16 h at 28°C as described previously (Alvarez et al., 2008).

Measurement of adenine nucleotide levels
Control and mutant epimastigotes were harvested and washed once with Buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, and 50 mM HEPES at pH 7.0). After washing, 1 × 10⁶ cells per treatment were centrifuged and resuspended in 100 μl of Buffer A and then lysed on ice for 30 min by the addition of 150 μl of 0.5 M HClO₄. The lysates were neutralized (pH 6.5) by the addition of 60 μl of 0.72 M KOH/0.6 M KHC₅O₃. Samples were centrifuged at 1000 × g for 5 min and the supernatant was separated for adenine nucleotide determination. ATP, ADP, and AMP in extracted samples were quantified by luciferin-luciferase bioluminescence assay in a plate reader as described (Huang et al., 2013). Briefly, we used an ATP Determination Kit according to manufacturer’s instructions with adenylate kinase and/or nucleoside-diphosphate kinase (NDK) (Sigma). To determine the amount of adenine nucleotides, four measurements were taken of three different reactions for each sample by endpoint determination of the ATP concentration: one reaction without the addition of any ATP-generating enzyme (for ATP), another reaction adding NDK (for ATP + ADP), and the third reaction adding both adenylate kinase and NDK (for ATP + ADP + AMP). The amount of ADP was obtained by subtracting the ATP value from the (ATP + ADP) value, and the amount of AMP was calculated from the difference between the (ATP + ADP + AMP) content and the (ATP + ADP) content.

Survival to H₂O₂ and C2-ceramide
To test the ability of TcMCUc-KO and TcMCUd-KO parasites to survive to oxidative stress, parasite cultures (2 ml LIT medium + 10% BFS) containing 1 × 10⁷ cells/ml were treated with 0 or 225 mM H₂O₂. After 4 h parasites were diluted 1/5 in 2 ml LIT medium + 10% BFS. The cells were counted after 72 h. The ability of TcMCUc-KO and TcMCUd-KO cells to survive to the apoptosis inducer C2-ceramide (N-acetyl-D-sphingosine) was tested by treating 2 × 10⁶ cells with 100 μM C2-ceramide for 20 h at 28°C. In both experiments, the results were expressed as the percentage of growth compared with untreated cultures. The cell numbers were determined in a cytometer chamber using the trypan blue dye to differentiate living and dead cells. The experiments were performed in triplicate.

Phylogenetic analysis
The phylogenetic tree shown in Supplemental Figure S1 was built with MEGA7 (Kumar et al., 2016) using the Neighbor-Joining method (Saitou and Nei, 1987) and the bootstrap method with 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992).
Statistical analysis

Statistical analyses were performed with GraphPad Prism software (La Jolla, CA), version 6.0. Reported values are means ± SD of n biological experiments, as indicated in the figure legends. The level of significance was evaluated by Student's t test for comparisons between two cell lines, one-way analysis of variance (ANOVA) for comparisons between more than two cell lines, and two-way ANOVA with multiple comparisons for analyses of grouped data.

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