1 **TITLE**

2 **Heme A synthesis and C*c*O activity are essential for *Trypanosoma cruzi* infectivity and**

3 **replication**

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5

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22 **ABSTRACT**

23 *Trypanosoma cruzi*, the causative agent of Chagas disease, presents a complex life cycle

24 and adapts its metabolism to nutrients availability. Although *T. cruzi* is an aerobic

25 organism, it does not produce heme. This cofactor is acquired from the host, and is

26 distributed and inserted into different heme-proteins such as respiratory complexes in the

27 parasite mitochondrion. It has been proposed that *T. cruzi´s* energy metabolism relies on a

28 branched respiratory chain with a cytochrome *c* oxidase type *aa*3 (C*c*O) as the main

29 terminal oxidase. Heme A, the cofactor for all eukaryotic C*c*O, is synthesized *via* two

30 sequential enzymatic reactions catalyzed by heme O synthase (HOS) and heme A synthase

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31 (HAS). Previously, TcCox10 and TcCox15 were identified in *T. cruzi*. They presented HOS
32 and HAS activity, respectively, when were expressed in yeast. Here, we present the first
33 characterization of TcCox15 in *T. cruzi*, confirming its role as heme A synthase. It was
34 differentially detected in the different *T. cruzi* stages, being more abundant in the
35 replicative forms. This regulation could reflect the necessity of more heme A synthesis, and
36 therefore more C*c*O activity at the replicative stages. Over-expression of a non-funtional
37 mutant caused a reduction in heme A content. Moreover, our results clearly showed that
38 this hindrance in the heme A synthesis provoked a reduction on C*c*O activity and, in
39 consequence, an impairment on *T. cruzi* survival, proliferation and infectivity. These
40 evidences support that *T. cruzi* depends on the respiratory chain activity along its life cycle,
41 being C*c*O an essential terminal oxidase.
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43

44 **SUMMARY STATEMENT**

45 *Trypanosoma cruzi* requires heme A as cofactor for the cytochrome *c* oxidase (C*c*O) of the
46 mitochondrial respiratory chain. The impairment of heme A synthesis negatively affects
47 parasite proliferation and infectivity, confirming its essentiality in the parasite life-cycle
48 stages.

49

50 **SHORT TITLE**

51 Heme A synthesis is essential for *Trypanosoma cruzi*

52

53 **KEYWORDS**

54 *Trypanosoma cruzi*, heme A synthase, heme A, cytochrome *c* oxidase, Chagas disease

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56 **ABBREVIATIONS LIST**

57 C*c*O, cytochrome *c* oxidase; DAPI, 4',6-diamidine-2-phenylindole; DMEM, Dulbecco's
58 Modified Eagle Medium; FBS, Fetal Bovine Serum; GFP, green fluorescence protein; Glc,

59   Glucose; Gly–EtOH, Glycerol–Ethanol; HAS, heme A synthase; HOS, heme O synthase; LIT,

60   Liver Infusion Tryptose; MOI, multiplicity of infection; PBS, phosphate buffered saline; SC-

61   URA, synthetic complete medium lacking Uracile; TcCox15, *Trypanosoma cruzi* Cox15

62   protein; YP, yeast extract, peptone, wt, wild type.

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64

65   **INTRODUCTION**

66   Heme (heme B) is an essential molecule for most aerobic organisms of all

67   kingdoms, and it is the prosthetic group of nearly all the heme-proteins including

68   hemoglobin, myoglobin, catalases, peroxidases and mitochondrial respiratory complexes

69   (1–3). It is synthesized *via* a well-defined pathway that is highly conserved through

70   evolution (2,4,5), where the last step – the insertion of ferrous iron into the

71   protoporphyrin IX ring - is catalized by ferrochelatase. Heme B can also be converted into

72   heme C or A, which contain different modifications in the protoporphyrin ring (2). Heme A,

73   B and C are cofactors in complexes II, III and IV of the respiratory chain as well as in

74   cytochrome *c*. Cytochromes *c* and *c*1 are examples of heme-proteins that contain heme

75   covalently bound to its apo-protein partner through its vinyl side chains (heme C), and

76   different mechanisms and enzymes were described for this type of attachment (6). The

77   only known protein which has heme A as a cofactor is the cytochrome *c* oxidase (C*c*O) or

78   complex IV of the mitochondrial respiratory chain (3,7). Heme A is synthesized *via* two

79   sequential enzymatic reactions starting from heme B. In the first reaction, catalyzed by the

80   heme O synthase (HOS) or Cox10, a farnesyl group is transferred to the C-2 vinyl group of

81   heme B to form heme O. During the second reaction, the methyl substituent on pyrrole

82   ring D is oxidized to an aldehyde by the heme A synthase (HAS), or Cox15, to form heme A

83   (Figure 1). Sequence analyses and biochemical studies have revealed that these enzymes

84   are highly conserved among eukaryotes and are located in the mitochondrial inner

85   membrane (3,7,8).

86   Trypanosomatids belong to the narrow group of eukaryotic organisms that are not

87   capable of synthesizing heme and, to overcome this deficiency, they extract this cofactor

88   from their environment. The mentioned group includes several organisms that cause

89 significant human and animal diseases worldwide, among them species of *Trypanosoma*

90 and *Leishmania*. *Trypanosoma cruzi* causes Chagas disease, the most prevalent parasitic

91 disease in the Americas (4,9). It is estimated that about 6 to 7 million people are infected

92 worldwide, mostly in Latin America and southern states of USA, where Chagas disease is

93 considered endemic. Moreover, it is also becoming relevant in non-endemic regions due to

94 migrations and the absence of control in blood banks and organ transplantation

95 (http://www.who.int/mediacentre/factsheets/fs340/en/) (10). This organism has a

96 complex life cycle that alternates between two hosts, an invertebrate and a vertebrate,

97 and involves at least four different stages. In the insect vector, two distinct forms were

98 described: the epimastigote, which is the replicative form, and the metacyclic

99 trypomastigote, the infective and non-replicative form that once in the mammals' blood

100 stream can infect any nucleated cell. In the mammal host two stages are undoubtedly

101 described: the amastigote (intracellular and replicative) and the bloodstream

102 trypomastigote (infective and non-replicative) forms. The last one is derived from the

103 intracellular amastigotes and released into the blood stream (11). During the different life-

104 cycle stages, the parasite adapts its metabolism to the nutrient availability inside the

105 different hosts. It is postulated that *T. cruzi*'s energy metabolism relies on the respiratory

106 chain, at least in some stages of its life cycle (12–14). It has also been proposed that a

107 branched respiratory chain is functioning in the parasite mitochondrion, with a type *aa*3

108 C*c*O as its main terminal oxidase, and with a contribution to the total oxygen consumption

109 of a putative alternative oxidase, as found in *T. brucei* (TAO), and/or an oxidase containing

110 cytochrome *o* (15–17)*.* However, other putative terminal oxidases were not identified in *T.*

111 *cruzi,* neither from its genome sequence (18), nor from proteomic analysis that reported

112 the presence of polypeptides belonging to mitochondrial complexes such as NADH

113 deshydrogenase, cytocrome *c*1, subunits of C*c*O, and ATP synthase complexes (19,20).

114 Although *T. cruzi* lacks the complete route for heme biosynthesis, several studies

115 have been demonstrated that it is essential for this parasite. It is capable of acquiring

116 heme from its hosts (21,22) and inserting it in different heme-proteins. Also, heme is

117 transported to the parasite mitochondrion where the cytochromes *c* and *c*1 are assembled

118 by a mechanism not yet characterized (9), and heme A is synthetized and inserted into the

119 mitochondrial C*c*O. In our laboratory we identified two *T. cruzi* proteins, named TcCox10

120 and TcCox15, which presented hemo O synthase and heme A synthase activity,

121 respectively, when they were expressed in yeast (23). Nevertheless, up to date, there is no

122 evidence about the role of TcCox10 and TcCox15, and the heme A synthesis in *T. cruzi.*

123   In this work, we present the first characterization of TcCox15, the HAS enzyme,

124 along the different life-cycle stages of *T. cruzi*. We focus on TcCox15's relevance to *T.*

125 *cruzi*'s survival and infectivity analyzing the expression of different versions of the TcCox15

126 recombinant protein -mutants and tagged ones- along the different life-cycle stages. Our

127 results demonstrate that TcCox15 has heme A synthase activity in *T. cruzi*, and that heme

128 A synthesis, and consequently the C*c*O activity, is essential in epimastigotes growth,

129 trypomastigotes infectivity and amastigotes intracellular replication.

130

131 **MATERIALS AND METHODS**

132 **Reagents.** Dulbecco's Modified Eagle Medium (DMEM) was obtained from Life

133 Technologies, Fetal Bovine Serum (FBS) from Natocor SA (Córdoba, Argentina), and hemin

134 from Frontier Scientific (Logan, UT, USA). Hemin stock solution was prepared in 50% (v/v)

135 EtOH, 0.01 N NaOH at a concentration of 1 mM, fractionated and stored at -80 °C.

136 **Bacterial and yeast strains.** *Escherichia coli* strains used for cloning procedures were

137 grown at 37 °C in Luria Bertani medium, supplemented with either ampicillin (100 μg/ml)

138 or kanamycin (50 μg/ml). *Saccharomyces cerevisiae* cells were grown in either a rich YP

139 medium (1% yeast extract, 2% peptone) or a synthetic complete medium (SC) lacking

140 Uracile for plasmid selection (SC-URA). In both cases, 2% Glucose (Glc) or 3% Glycerol–2%

141 Ethanol (Gly–EtOH) were used as carbon sources. *S. cerevisiae* strains DY5113 (MATa *ade2-*

142 *1 his3-1,15 leu2-3,112 trp1Δ ura3-1*) and *cox15Δ* (DY5113 *cox15::KanMX4*, (23)) were used

143 for heterologous complementation assays. Yeast cells were transformed using the lithium

144 acetate method (24).

145 **Cell lines and parasites.** Vero cells (ATCC CCL-81, already available in our laboratory) were

146 maintained in DMEM supplemented with 0.15% (w/v) NaHCO$_3$, 100 U/ml penicillin, 100

147 μg/ml streptomycin and 10% heat inactivated FBS (DMEM-10%FBS) at 37 °C in a humid

148 atmosphere containing 5% CO$_2$. *T. cruzi* epimastigotes Dm28c strain were cultured in a

149 Liver Infusion Tryptose (LIT) medium supplemented with 10% heat inactivated FBS (LIT-

150 10%FBS) and 20 µM hemin (25), at 28 °C. Metacyclic trypomastigotes were obtained by

151 spontaneous differentiation of epimastigotes at 28 °C and used to infect monolayered

152 Vero cells to obtain cell-derived trypomastigotes. After two rounds of infection, the

153 resulting cell-derived trypomastigotes were used to infect monolayered Vero cells at a

154 multiplicity of infection (MOI) of 10-30 to monitor the cell infection experiments, or the

155 development and replication of intracellular amastigotes. During *T. cruzi* infections,

156 monolayered Vero cells were incubated in DMEM-2%FBS (2% of heat inactivated FBS) at

157 37 °C in a humid atmosphere containing 5% CO_2.

158 *In silico* **analysis.** The protein sequences: TcCox15 (TCDM_06426, obtained from TriTrypDB

159 database, http://tritrypdb.org/tritrypdb/ (26)), the *S. cerevisiae* ScCox15 (YER141W,

160 obtained from the *Saccharomyces* Genome Database http://www.yeastgenome.org), and

161 the *B. subtilis* CtaA (CAB11340.1, obtained from NCBI http://www.ncbi.nlm.nih.gov) were

162 used for the amino acids' multiple sequence alignments using the Clustal W and Clustal X

163 version 2.0 software (http://www.ebi.ac.uk/Tools/msa/clustalw2, (27)).

164 **Ethics statement**. All experiments were approved by the Comité Institucional para el

165 Cuidado y Uso de Animales de Laboratorio, Facultad de Ciencias Bioquímicas y

166 Farmacéuticas, Universidad Nacional de Rosario, Argentina (Institutional Committee of

167 Animal Care and Use of the School of Biochemical and Pharmaceutical Sciences, National

168 University of Rosario Argentina) and conducted according to specifications of the US

169 National Institutes of Health guidelines for the care and use of laboratory animals (File

170 number 935/2015).

171 **Polyclonal antibodies that recognize TcCox15.**

172 To obtain anti-TcCox15 antibodies, we used the P1TcCox15 peptide expressed as a GST-

173 fusion protein. Experimental details are included in the Supporting Information. The rabbit

174 polyclonal antibodies were obtained from the Animal Facility, Facultad de Ciencias

175 Bioquímicas y Farmacéuticas - Universidad Nacional de Rosario. Animals were housed and

176 maintained according to institutional guidelines.

177 **TcCox15.His-GFP fusion protein.** The *TcCOX15.HIS-GFP* recombinant gene was made in a

178 two-step procedure. First, the *TcCOX15.HIS* sequence was amplified by PCR, using the

179   pBluescriptIIKS(-).*TcCOX15.HIS* vector as a template (23), and FP_*TcCOX15* and RP_noStop

180   primers (Table S1) to eliminate STOP codon at its 3'-end; *Bam*HI and *Xho*I restriction sites

181   flanked the *TcCOX15.HIS-nonSTOP* sequence. Also the primers FP_*GFP* and RP_*GFP,* listed

182   in Table S1, were designed to amplify GFP sequence from a plasmid available in the

183   laboratory introducing the *Xho*I restriction site at the 5´-end and *Sal*I *Eco*RV *Xba*I at the 3´-

184   end. The GFP PCR product was treated with the *Xho*I and *Xba*I restriction enzymes and the

185   *TcCOX15.HIS-nonSTOP* was treated with *Bam*HI and *Xho*I enzymes. *TcCOX15.HIS-nonSTOP*

186   genes and the GFP gene were cloned together into a pENTR^{TM}3C vector (Gateway system®,

187   Invitrogen) to form *TcCOX15.HIS-GFP*. The cloning procedure was verified by sequencing

188   the DNA insert. After that, *TcCOX15.HIS-GFP* was cloned in p426.M25 vector previously

189   used in our laboratory for yeast complementation assays (23) and in pTcINDEX vectors

190   (28) for *T. cruzi* expression assays.

191   ***TcCOX15.HIS* mutant genes.** The mutants *TcCOX15H129A*, *TcCOX15H206A* and

192   *TcCOX15H307A* were made following the site directed mutagenic method described by

193   Oded Edelheit with some modifications (29). The mutagenic primers are listed in Table S1.

194   The pENTR3C.*TcCOX15.HIS* vector obtained from *E. coli* DH5α (*dam*$^+$) was used as a

195   template. The reaction mixture (100 µl) contained 0.3 mM of each dNTP, 0.2 µM of each

196   mutagenic primer, 400 ng of template DNA, 1 mM MgSO$_4$, 10 µl of the 10X buffer

197   (Invitrogen) and 2.5 units of *Pfx* Platinum DNA polimerase (Invitrogen). The reaction steps

198   were: one cycle of 3 minutes at 95 °C, then 18 cycles of 30 seconds at 95 °C, 1 minute at 55

199   °C and 5 minutes at 68 °C. After that, the PCR mix was treated with 20 U of *Dpn*I

200   (Fermentas) at 37 °C for 2 hours where the methylated DNA used as template was

201   completely digested. 10 µL of 1 M CaCl$_2$ were added to 90 µL of the mixture and then used

202   to transform *E. coli* DH5α cells by the chemical method. The plasmids containing the

203   mutated sequences were identified by restriction enzyme treatment since a specific

204   restriction site was introduced together with the aminoacid substitution by the mutagenic

205   primer (30). The mutations were confirmed by DNA sequencing, and the inserts cloned to

206   pTcINDEX vector (28) for *T. cruzi* expression assays and also between *Bam*HI and *Sal*I

207   restriction sites in p426.MET25 vector for yeast complementation assays (23).

208   **Spot growth assay.** *S. cerevisiae* wt and *cox15Δ* cells transformed with plasmids derived of

209   p426.MET25 were grown overnight in SC–URA medium at 30 °C with vigorous shaking.

210 Then, the cells were suspended at a final D^{600} = 1 and 5 μl of four serial dilutions (1:10 to

211 1:10000) plated in solid SC-URA Glc and SC-URA Gly-EtOH. When it was necessary, the

212 methionine concentration was modified to evaluate the effect caused by different amount

213 of overexpressed recombinant protein, since the *MET25* promoter is repressed by

214 methionine (31). The plates were incubated at 30 °C for 4–5 days.

215 **Parasites transfection.** *T. cruzi* epimastigotes Dm28c containing pLEW13 plasmid (28)

216 (Dm28c.pLEW13), were grown in LIT-10%FBS with 20 μM hemin plus 250 μg/ml G418 to

217 approximately 3×10^7 cells/ml. Epimastigotes were collected by centrifugation at 3000 × g

218 for 5 minutes, washed twice with PBS, and 1×10^8 cells were suspended in 0.35 ml of

219 electroporation buffer pH 7.5 (137 mM NaCl, 2.7 mM KCl, 4.7 mM Na_2HPO_4, 1.47 mM

220 KH_2PO_4, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$). The cells were transferred to a 0.2 cm gap cuvette

221 and 50 μl of 0.4 μg/μl DNA was added (wt and mutant genes cloned into pTcINDEX

222 plasmid). The mixture was placed in ice for 15 min and subjected to 1 pulse of 450 V and

223 500 μF using GenePulser II (Bio-Rad, Hercules, USA). Then, it was transferred to 3 ml of LIT-

224 10%FBS with 20 μM hemin and incubated at 28 °C. 48 hours post electroporation 50 μg/ml

225 of Hygromycin B was added, and at 72 to 96 hours, the cultures were diluted and

226 Hygromycin B concentration was increased to 200 μg/ml. Stable resistant transfected cells

227 were obtained approximately 30-45 days post transfection.

228 **Epimastigotes' growth curves.** Epimastigotes transfected with pTcINDEX plasmids

229 containing the wt or mutant *TcCOX15* genes were maintained in LIT-10%FBS plus 20 μM

230 hemin. They were collected by centrifugation, and 5×10^6 cells were suspended in 1.5 ml

231 of fresh medium. After 24 hours of growth, tetracycline was added at different

232 concentrations (0.05, 0.15 and 0.25 μg/ml) to induce gene expression and the cultures

233 were maintained for 7-10 days in mid-log phase by periodic dilutions every two days. Cell

234 growth was monitored by cell counting in a Neubauer chamber. Samples were taken 3-4

235 days post induction to analyze protein expression by Western blot assay and indirect

236 immunofluorescence method, heme A levels by the heme pyridine hemochrome method,

237 and C*c*O activity by oxygen consumption rates measurement. In all cases, the accumulation

238 of the recombinant proteins caused by the addition of different amount of tetracycline

239 was evaluated by Western blot assay. Recently, it was reported by Moullan and col. that

240 tetracycline can disturb mitochondrial function in different eukaryotic models (32). On the

241 other hand, Hashimi and col. reported later that higher tetracycline concentrations did not

242 affect *T. brucei* and *L. tolerantoae* (33), but they did not test the effect on *T. cruzi* (32,33).

243 Considering the presented evidences, and to avoid other possible effects caused by the

244 mitochondrial protein imbalance, we performed the assays using the lower inductor

245 concentration that caused a phenotypic difference between the epimastigotes containing

246 the recombinant proteins (wt and mutants) and the controls.

247 **Cell infection assay using trypomastigotes overexpressing the recombinant *TcCOX15* wt**

248 **and mutant genes.** Cell-derived trypomastigotes (containing pTcINDEX,

249 pTcINDEX.*TcCOX15.HIS* and pTcINDEX.*TcCOX15H307A.HIS*) were used to infect

250 monolayered Vero cells to evaluate the effects of (wt and mutant) TcCox15 recombinat

251 protein during tripomastigotes´ infection and intracellular amastigotes' replication (2-3

252 days post infection) as it was previously described (22,34). Briefly, the cell-derived

253 trypomastigotes were collected by centrifugation at 4,000 x g for 10 minutes and pre-

254 incubated in both, the absence (-) and the presence (+) of 0.20 µg/ml tetracycline for 1 h.

255 Then they were incubated during 16 hours with 3 x 10^3 Vero cells plated on cover glasses

256 (mantained in DMEM-2%FBS at 37 °C in a humid atmosphere containing 5% CO_2) at a MOI

257 (multiplicity of infection) of 10-30 trypomastigotes/cell (Infection), Then, the monolayered

258 Vero cells were washed twice with PBS, the complete medium was renewed without (-) or

259 with (+) 0.20 µg/ml tetracycline and then incubated during 2 - 3 days (post infection). After

260 that, the treated monolayered cells were washed twice with PBS, fixed with methanol for

261 15 minutes, stained with Giemsa reagent and mounted with Canada Balsam (Biopack). To

262 quantify the percentage of infected cells and the number of amastigotes per infected cell,

263 200 cells per slide were analyzed by optical microscopy. All conditions were run by

264 triplicate (technical replicates) and the results are representative of at least two

265 independent assays (biological replicates).

266 **Yeast mitochondrial purification**. Intact yeast mitochondria were isolated from yeast

267 grown in a synthetic medium as previously described (23). The final pellet containing the

268 crude mitochondrial fraction was suspended in a Spheroplast buffer (0.6 M Manitol, 20

269 mM Tris:HCl pH 7.5, 1 mM PMSF), immediately used or fractioned and stored at -80 °C.

Total protein cell extracts obtained from different life-cycles stages of *T. cruzi*. Epimastigotes maintained in exponential growth phase in LIT-10%FBS with 20 µM hemin (plus tetracycline in case of transfectant epimastigotes) were collected by centrifugation at 2000 x g, washed twice with PBS, and suspended in a Lysis buffer (8 M urea, 20 mM Hepes pH 8). The cell-derived trypomastigotes were collected by centrifugation at 6000 x g for 10 minutes, washed twice with PBS, and suspended in the same Lysis buffer. Amastigotes were obtained from infected monolayered Vero cells maintained in DMEM-2%FBS. 48 hours post infection, the monolayered cells were washed twice with cold PBS and lifted with a cell scraper in 5 ml of PBS. The cells were collected by centrifugation at 1500 x g for 8 minutes and suspended with 1 ml of PBS, and then disrupted by a 27G syringe. The amastigotes were collected by centrifugation at 1500 x g for 8 minutes and suspended in Lysis buffer.

Immunoblotting. Total protein from *T. cruzi* cell-free extracts (10-15 x 10^6 cells/well) or *S. cerevisiae* mitochondrial fractions (30-50 µg protein per well) were heated at 50 °C for 15 minutes in a loading buffer (1.8% SDS, 5% glycerol and 0.5% β-mercaptoethanol), separated by electrophoresis on a 12-15% SDS-PAGE, and electrotransferred onto nitrocellulose membranes (Amersham). The following antibodies were used for protein detection: rabbit polyclonal anti-TcCox15 antibody, rabbit monoclonal anti-GFP antibody (Santa Cruz Biotechnology), mouse monoclonal anti-His antibody (Calbiochem) or rabbit polyclonal anti-6xHis HRP conjugated antidody (ABCAM), mouse monoclonal anti-trypanosome α-tubulin clone TAT-1 antibody (a gift from K. Gull, University of Oxford, England, United Kingdom), rabbit polyclonal anti-mitochondrial tryparedoxin peroxidase antibody (anti-TcmPx, kindly provided by Sergio Guerrero, Universidad Nacional del Litoral, Argentina) (35), and rabbit polyclonal anti-β subunit of F1 ATPase complex antibody (anti-subβ F1, a kind gift from A. Tzagollof, Columbia University, USA). Bound antibodies were detected with peroxidase-labeled anti-rabbit IgG (Calbiochem) or peroxidase-labeled anti-mouse IgG (Calbiochem) and ECL Prime Western Blotting Detection kit (GE Healthceare). When it was necessary, the membranes were stripped 30 minutes at 50 °C with Stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris pH 8). The ImageJ software was used for the quantitative analysis (36,37).

Indirect immunofluorescence. Epimastigotes maintained in an exponential growth phase in LIT-10%FBS with 20 µM hemin (plus the addition of tetracycline when necessary) were collected and 10 x 10^6 cells were incubated with 1 µM Mitotracker (Invitrogen) in 100 µL of PBS during 60 minutes at 28 °C. The cells were fixed with 4% (w/v) paraformaldehyde in PBS at room temperature for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Then they were incubated first with rabbit anti-TcCox15 polyclonal antibody diluted in Immunofluorescence Buffer (IB - 0.1% Tween 20, 150mM Tris pH 7.5 and 150mM NaCl) plus 1 % BSA, washed, and later incubated with anti-rabbit IgG FitC conjugated (Jackson Immuno Research) diluted in the same IB and 1 µM DAPI (4',6-diamidine-2-phenylindole). The slides were mounted with VectaShield (Vector Laboratories). Images were acquired with a confocal Nikon Eclipse TE-2000-E2 microscope using Nikon EZ-C1 software. The ImageJ software was used to process the images (36,37). The autofluorescent background was rectified and the deconvolution treatment was applied to all images. The co-localization quantitative analysis was performed using the Image J software; the estimated Pearson's correlation coefficient and Manders' overlap coefficients are reported.

Oxygen consumption measurements. The oxygen consumption rates were quantified using a Clark electrode connected to a 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co.) from the linear response as previously described (23). The yeast cells grown overnight in SC–URA Glc were collected, washed, and suspended in 3% glycerol (v/v). The measurements of oxygen consumption rate (O_2 nmoles consumed per minute and D^{600}) were carried out in 3% glycerol (v/v) at 30 °C in a final volume of 2 ml. The reaction was completely inhibited by the addition of azide (a few grains).

The oxygen consumption rate of transfected epimastigotes (O_2 nmoles consumed by 10^6 cells per minute) was measured following the protocol previously described by Vercesi and col. (38) with minor modifications. Epimastigotes transfected with pTcINDEX containing the *TcCOX15* genes (wt and mutants) were treated with tetracycline for 3-4 days, then collected, washed with PBS, suspended at 50 x 10^6 cells/100 µL in TSB-EGTA (125 mM sucrose, 65 mM KCl, 10 mM Tris:HCl pH 7.5, 1 mM $MgCl_2$, 2.5 mM KH_2PO_4, 333 µM EGTA) and kept in ice. 5 mM succinate and 50 µM digitonin were added to each sample

330   immediately before the assay. The measurements of oxygen consumption were carried

331   out in TSB-EGTA plus 5 mM succinate at 28 °C in a final volume of 2 ml.

332   **Heme pyridine hemochrome method.** 2-4 x 10^9 cells of transfected epimastigotes were

333   collected after 3-4 days post induction with tetracycline, washed with PBS, and pulled

334   down by high-speed centrifugation discharging the supernatant. Total hemes were

335   extracted from the cell pellet by the acetone:HCl extraction method previously described

336   with minor modifications (39). Briefly, the cell pellet was washed with 1 ml of cold

337   acetone:water (80:20) and then mixed with 1 ml of acetone:HCl (4 vol acetone and 1.5 vol

338   1.5 M HCl) to extract heme A and B. Then, the sample was centrifuged and the

339   supernatant was kept in a separated tube. Hemes were extracted again from the pellet

340   with 0.5 ml of acetone:HCl. Then, both supernatants were combined and mixed with 1 ml

341   of ethyl ether and 0.25 ml of 1.5 M HCl to extract hemes from the acetone:HCl solution.

342   The last step (ether extraction) was repeated but the extraction was performed with 0.5

343   ml of ethyl ether and 0.125 ml of 1.5 M HCl. The ethyl ether fractions were combined and

344   washed with 0.8 ml of 0.5 M HCl, and with 0.8 ml of 3% (w/v) NaCl. After ethyl ether

345   evaporation, the extracted hemes were suspended with 500 μL PBS and their

346   concentrations quantified from the reduced – oxidized differential spectrum as described

347   previously (22). The 557 nm from heme B and 588 nm from heme A absorbance peaks

348   were identified spectrum and heme B and heme A concentrations were quantified using

349   the molar extinction coefficient 23.98 mM^{-1} cm^{-1} and 25.02 mM^{-1} cm^{-1}, respectively (39).

350   **Statistical analysis.** The results are expressed as mean ± S.D. of three technical replicates

351   (technical replica) from one representative of three independent experiments (biological

352   replica) unless otherwise indicated. Statistically significant differences between groups (P)

353   were analyzed using GraphPad Prism version 6.0 as indicated in each assay.

354

355   **RESULTS**

356   **TcCox15 is detected in the different life-cycle stages of *Trypanosoma cruzi***

357   To study the role and relevance of TcCox15 in *T. cruzi*, first we evaluated its

358   presence along the parasite's life cycle. The endogenous protein was detected in all

359   assayed life-stages as shown in Figure 2, where anti-TcCox15 antibodies were used in the

360   Western blot assay. The replicative life-stages (epimastigotes and amastigotes) showed

361   higher TcCox15 levels than the infective and non-replicative stage (trypomastigotes). The

362   TcCox15 signal was normalized against tubulin or TcmPx, revealing the amount of this

363   protein was two times higher in epimastigotes than in amastigotes and five times higher

364   than in trypomastigotes (Figure 2B and C). These results suggest a major requirement for

365   HAS activity in the replicative stages.

366   **TcCox15 is detected in the mitochondrion of *Trypanosoma cruzi* epimastigotes.**

367   Indirect immunofluorescent assay on epimastigotes (Dm28c) were run to assess the

368   cellular localization of TcCox15 (using anti-TcCox15 antibodies), but the resulting

369   fluorescent signals presented very low intensity, impeding a clear identification of the

370   protein location. Alternatively, transfected epimastigotes containing the

371   pTcINDEX.*TcCOX15.HIS*, treated with tetracyclin during 3 days to induce the recombinant

372   gene expression, were analyzed. Anti-TcCox15 as the primary antibody and the anti-rabbit

373   FitC-conjugated as the secondary antibody allowed the detection of the recombinant

374   TcCox15.His. Also the samples were labeled with Mitotracker. In this case, the green

375   fluorescent signal corresponding to TcCox15.His overlapped with the Mitotracker's red

376   signal, as shown in Figure 2D, suggesting that TcCox15 localizes in the parasite's

377   mitochondrion, as it was expected.

378   **TcCOX15.His-GFP fusion is dominant negative**

379   In addition to TcCox15.His we also constructed a recombinant protein fused to a C-

380   terminal GFP where the *TcCOX15.HIS* gene was C-terminally tagged with a GFP sequence

381   generating the *TcCOX15.HIS-GFP* recombinant gene. The functionality of TcCox15.His-GFP

382   was evaluated in *cox15Δ* yeast cells. Then wt and *cox15Δ* yeast cells were transformed

383   with *TcCOX15.HIS-GFP* cloned in pRS426.MET25, and plated on selective media

384   supplemented with Glc or Gly-EtOH to test the respiratory capacity. The expression of

385   TcCoxX15.His-GFP did not fully restore the respiratory deficiency of the knocked out

386   *cox15Δ* cells as it is shown in Figure 3A. Moreover, the oxygen consumption of *cox15Δ* cells

387   over-expressing TcCox15.His-GFP was lower than cells expressing TcCox15.His (Figure 3B).

388   The presence of both recombinant proteins was corroborated by Western blot assays

389 using anti-TcCox15 and anti-GFP antibodies (Figure 3C), and also TcCox15.His-GFP was

390 visualized in the yeast cells by confocal microscopy (Figure 3D). Surprisingly, the over-

391 expression of the recombinant protein TcCox15.His-GFP, contrary to TcCox15.His, affected

392 negatively the respiratory growth of the wt cells (Figure 3E). Presumably, this dominant

393 negative effect was due to a defective formation of the complexes necessary for heme A

394 synthesis and/or heme A insertion into subunit I of CcO (40,41). In addition to this

395 evidence, *TcCOX15.HIS-GFP* was also cloned in pTcINDEX (28) and the resulting plasmid

396 used to transfect *T. cruzi* epimastigotes (Dm28c.pLEW13). It was not possible to obtain

397 transfectant epimastigotes harboring pTcINDEX.*TcCOX15.HIS-GFP*, consistently with the

398 phenotype observed when *TcCOX15.HIS-GFP* was expressed in wt yeast.

399 **The conserved histidine residues of TcCox15 enzyme are essential for heme A synthase**

400 **activity**

401 Several His residues were described as essential for the CtaA activity (*B. subtilis* HAS

402 enzyme), such as His60, His123, and His216 (42). These residues are conserved in TcCox15,

403 corresponding to His129, His206 and His307 following the TcCox15 numbering

404 (Supplementary Figure S1) (23). To analyze their relevance, each one was changed by Ala

405 generating the TcCox15HxxxA.His (H129A, H206A and H307A) mutant proteins (all of them

406 containing a C-terminal His-tag). The HAS activity of these recombinant proteins was

407 evaluated in yeast cells, then the *TcCOX15HxxxA.HIS* variants cloned in p426.MET25

408 (p426.MET25.*TcCOX15H129A.HIS*,   p426.MET25.*TcCOX15H206.HIS*   and

409 p426.MET25.*TcCOX15H307A.HIS*) were used to transform the *cox15Δ* cells as well as

410 p426.MET25   (negative   control),   and   p426.MET25.*TcCOX15.HIS*   and

411 p426.MET25.*ScCOX15.HIS* as positive controls (23). The presence of TcCox15H129A.His,

412 TcCox15H206A.His and TcCox15H307A.His did not allow the recovery of the respiratory

413 growth of *cox15Δ* cells, and also the recombinant strains did not exhibit any oxygen

414 consumption activity as it is shown in Figure 4A and B respectively. The presence of the wt

415 TcCox15.His and the TcCox15HxxxA.His mutants in mitochondrial fractions was verified by

416 Western blot assays (Figure 4C). In summary, the respiratory deficiency was not due to the

417 absence of these recombinant proteins in the yeast mitochondria but instead to the

418 mutations introduced in TcCox15 sequence. Also, the phenotype caused by the over-

419 expression of TcCox15HxxxA.His was evaluated in wt yeast cells and its growth was not

420   affected like it was observed with the over-expression of the TcCox15.His-GFP. This

421   suggests that TcCox15HxxxA.His mutants did not affect the activity of the native HAS

422   enzyme in yeast (ScCox15). These results show that the mutations introduced in the

423   *TcCOX15* gene affected the enzyme function without major effect on its assembly and

424   accumulation in the yeast mitochondria, which makes them promising candidates to

425   evaluate their function in the native organism, *T. cruzi*.

426   **The presence of TcCox15 mutant negatively affects heme A synthesis, C*c*O activity and**

427   **epimastigotes proliferation**

428   As an approach to analyze the role of TcCox15 in *T. cruzi*, *TcCOX15HxxxA.HIS* (H129,

429   H206 and H307) genes were cloned in pTcINDEX vector and pTcINDEX.*TcCOX15H129A.HIS*,

430   pTcINDEX.*TcCOX15H206A.HIS*,   pTcINDEX.*TcCOX15H307A.HIS*,   in   addition   to

431   pTcINDEX.*TcCOX15.HIS*, were used to transfect epimastigotes Dm28c.pLEW13 (28). We did

432   not succeed in obtaining epimastigotes transfected with pTcINDEX.*TcCOX15H206A.HIS,* so

433   we decided to exclude this mutant for the studies conducted afterwards. We tested the

434   phenotype caused by the induction of the recombinant genes (*TcCOX15.HIS* and

435   *TcCOX15HxxxA.HIS*) by the addition of different amounts of tetracycline, from 0 to 0.25

436   µg/ml. In this case, we did not observe any effects in the controls (epimastigotes harboring

437   pTcINDEX) up to 0.5 µg/ml of tetracycline (Figure 5A, B and C) and the induction of the wt

438   gene expression (*TcCOX15.HIS*) caused a mild negative effect on growth when the

439   concentration of tetracycline was increased from 0.15 to 0.25 µg/ml (Figure 5A). However,

440   the induction of both mutant genes severely impaired the epimastigotes' growth at the

441   lowest tetracycline concentration assayed (0.15 µg/ml) as shown in Figure 5B and C. The

442   effect produced by the presence of recombinant proteins was reported as a percentage of

443   parasite number of induced/non-induced conditions at the end of the growth curve, day 8

444   (Figure 5D). The accumulation of the recombinant proteins was validated by Western blot

445   assays (Figure 6A). Also, the cellular location of the mutant proteins (TcCox15H129A.His

446   and TcCox15H307A.His) was verified by indirect immunofluorescence assays (Figure 6B),

447   where the signal corresponding to the TcCox15HxxxA.His overlapped the mitochondrial

448   marker signal (Mitotracker), similarly to TcCox15 wt (Figure 2D), suggesting that the

449   mutations (H129A and H307A) did not affect the protein localization.

450   Afterwards, heme A synthesis and C*c*O activity were evaluated on epimastigotes

451   over-expressing TcCox15.His and TcCox15H307A.His recombinant proteins. Heme A

452   content was quantified by the pyridine hemochrome method (39) and C*c*O activity by

453   oxygen consumption measurements. The heme A content is reported in Table 1. All the

454   samples showed similar heme B concentrations, however, epimastigotes containing the

455   TcCox15H307A.His non-functional enzyme showed a 50% reduction in heme A

456   concentration, which is also denoted by the heme A/heme B ratio. Moreover, the parasites

457   containing the TcCox15H307A.His decreased about 40-45% the oxygen consumption rates

458   when compared to the non-induced samples (Figure 7). Conversely, parasites containing

459   TcCox15.His (wt protein) did not show any significant variations in the oxygen

460   consumption rates (Figure 7). Taking into account all results presented here, we can

461   postulate that the over-expression of the non-functional TcCox15 mutants caused a

462   negative effect over heme A synthesis, affecting the function of the C*c*O complex and

463   epimastigotes' proliferation.

464   The presence of the TcCox15 non-functional mutant negatively affects *T. cruzi* infection

465   (trypomastigotes) and intracellular replication (amastigotes)

466   We evaluated if any reduction in heme A synthesis and consequently C*c*O activity

467   could affect *T. cruzi* infection and intracellular replication. Cell-derived trypomastigotes

468   containing pTcINDEX, pTcINDEX.*TcCOX15.HIS* and pTcINDEX.*TcCOX15H307A.HIS* plasmids

469   pre-treated without or with 0.20 µg/ml of tetracycline, were incubated with monolayered

470   Vero cells at a ratio of 10 trypomastigotes/cell during 16 h at 37°C without or with the

471   addition of tetracycline (infection without (-), Infection with (+)). Then, the free

472   trypomastigotes were washed out, the complete medium was replaced without (-) or with

473   (+) the addition of 0.20 µg/ml of tetracycline and the cells incubated for another 2 days,

474   (post infection without (-), post infection with (+)). Then, the cells were stained with

475   Giemsa, and the number of infected cells and the intracellular amastigotes per infected

476   cells were evaluated microscopically. No effect was observed in parasites transfected with

477   the pTcINDEX (not shown). The presence of the recombinant TcCox15.His (wt enzyme) did

478   not affect the number of infected cells or the intracellular amastigotes per infected cells at

479   any of the assayed conditions, as shown in Figures 8A and B. Representative Giemsa

480   stained cells infected with *T. cruzi* expressing TcCox15.His are presented in Figure 8C. On

481 the other hand, the expression of TcCox15H307A.His impacted negatively on the number

482 of infected cells and the amount of amastigotes per infected cell (Figure 8D and E).

483 Induction of *TcCOX15H307A.HIS* expression during infection (+/- or +/+) caused a reduction

484 in the number of infected cells by approximately 50%, but no effect was observed when

485 the tetracycline had been added once the infection was established (-/+) (Figure 8D). Also,

486 a significant reduction in the number of amastigotes per infected cell was caused when the

487 tetracycline was added during the infection (+/-) or when it was maintained throughout

488 the whole assay (+/+). Aditionally, a lower number of amastigotes per infected cell was

489 observed when tetracycline was added once the infection was established (-/+), but the

490 effect was less severe (Figure 8E). A representative picture of infected cells with *T. cruzi*

491 over-expressing TcCox15H307A.His is presented in Figure 8F. Both recombinant proteins

492 (TcCox15.His and TcCox15H307A.His) were detected by Western blot assay from total cell-

493 free extracts of transfected trypomastigotes and amastigotes (Figure 8G). We also verified

494 that the impairment in heme A synthesis affected the earlier steps on the infection

495 processes, as it is described in supporting information, where the over-expression of

496 TcCox15H307A.His recombinant protein caused a reduction on the percentage of infected

497 cells at short periods of time post infection (Figure S4). Thus, the presence of the TcCox15

498 non-functional mutant that reduces the heme A synthesis impaired the trypomastigote

499 infection and the intracellular amastigote replication.

500

501 **DISCUSSION**

502   Heme A is an essential cofactor produced only for the mitochondrial respiratory

503 complex IV (C*c*O); its insertion into subunit I of this complex is one of the first stages in the

504 C*c*O assembly (43). It is reported that the impairment in heme A synthesis and/or its

505 proper insertion into subunit I causes the proteolysis of the core of C*c*O in yeast, resulting

506 in a failure to detect this complex in the mitochondrial inner membrane (3). Therefore, a

507 lack of success in the synthesis and correct loading of heme A leads to respiratory chain

508 defects. *T. cruzi,* like other trypanosomatids relevant for human health, are aerobic

509 organisms that rely, at least in some of their life-cycle stages, on the mitochondrial

510 respiratory chain activity. Acknowledging these pieces of evidence, we postulate that

heme A might be synthesized according to the requirements of assembled and active C*c*O, depending on the energetic metabolism of the *T. cruzi* life-cycle stages, nutrient availability, or both. Furthermore, it seems reasonable that a reduction, or inhibition, in heme A synthesis would render a defect on C*c*O activity that could lead to an increase in the other terminal oxidase activities to compensate the impairment of the C*c*O function.

In this work, we present the first characterization of the TcCox15 enzyme (HAS) and its product –heme A- in the native organism, *T. cruzi*. Our data indicates that TcCox15 should localize in the parasite mitochondrion, like all the eukaryotic HAS studied to date. Recently reported transcriptomic analysis described that genes belonging to the Krebs cycle, the respiratory chain and oxidative phosphorylation were significantly up-regulated in epimastigotes, however, expression of genes related to respiration were detected in all stages (44). Our results show that the expression of TcCox15 is regulated throughout the parasite life cycle, being more abundant in the replicative stages (epimastigotes and amastigotes) than in the infective stage (trypomastigotes), in agreement with the transcriptomic analysis. Moreover, the TcCox15 expression pattern could reflect the necessity of more respiratory chain activity during the replicative stages and to support this demand of C*c*O, more HAS activity is required.

Several His residues, reported as essential for HAS activity in other organisms, are conserved in the *T. cruzi* protein (23,42). Their replacement by Ala in TcCox15 rendered non-functional enzymes (TcCox15H129A, TcCox15H206A and TcCox15H307A), and together with the data reported from CtaA (*B. subtilis* HAS enzyme) (42), and recently from ScCox15 (*S. cerevisiae* Cox15 enzyme) (41), it confirmed the relevance of the conserved His residues in HAS activity. Also, the dominant negative effect observed when the recombinant protein TcCox15.His-GFP was expressed in wt yeast cells, as well as the inability to obtain transfected epimastigotes expressing this recombinant protein, suggests that the presence of the C-terminal GFP interferes with HAS activity. This strongly supports that TcCox15 could form relevant oligomeric complexes involved in heme A synthesis and/or heme A insertion into subunit I of C*c*O as it was described for other eukaryotic HAS (40,41). All these evidences indicate that heme A synthesis (at least the reaction catalyzed by HAS), and possibly the cofactor insertion into C*c*O, are conserved in *T. cruzi* despite the evolutionary divergence of this organism.

542   The non-funtional mutants of TcCox15 araise as a useful tool to analyze the role of

543   heme A and its synthesis in *T. cruzi*. Their expression in epimastigotes, which also contain

544   endogenous TcCox15, negatively affects parasite growth as a direct consequence of the

545   impairment of heme A synthesis and consequently C*c*O activity. *A priori*, we exclude any

546   other possibility since the over-expression of the recombinant wt enzyme did not affect

547   heme A synthesis, C*c*O activity and epimastigotes' growth. Besides, these recombinant

548   proteins (wt and mutants) were localized in the parasite mitochondrion and interestingly

549   their presence did not affect heme B concentration in epimastigotes. Recently, it was

550   postulated that yeast Cox15 (ScCox15) associates with the C*c*O assembly intermediates

551   during Cox1 maturation, where Cox15 itself plays a relevant role in heme A insertion into

552   Cox1 (40,41). Then, it is reasonable to postulate that non-functional TcCox15 caused a

553   dominant-negative effect on epimastigotes' growth, where the inactive enzyme could

554   replace some of the endogenous TcCox15, disrupting the relevant complexes involved in

555   heme A synthesis and/or heme A insertion into C*c*O. In spite of the negative effects caused

556   in C*c*O activity by the presence of the TcCox15 non-functional mutants, the epimastigotes'

557   oxygen consumption rates were not compensated by any other mechanism under our

558   assay conditions. Therefore, our results confirm that C*c*O is the main terminal oxidase

559   essential for the epimastigotes' proliferation.

560   The expression of the recombinant non-functional HAS (TcCox15H307A.His)

561   negatively affected the cell line infection produced by trypomastigotes, while the

562   recombinant wt protein did not cause any effect. Notably, the endogenous level of

563   TcCox15 in trypomastigotes (infective form) was the lowest detected compared to the

564   replicative forms. Then, any small amount of a recombinant protein (wt or non-functional

565   mutant) could produce a serious imbalance in the HAS activity and finally in the amount of

566   fully assembled C*c*O. It has been previously proposed that once the trypomastigotes enter

567   the cell they have to differentiate into amastigotes and escape from parasitophorous

568   vacuole to establish the infection (45), or leave the cell (46,47). Interestingly, our results

569   demonstrate that heme A synthesis and C*c*O activity are essential for the cellular infection

570   produced by the *T. cruzi* trypomastigotes, and supports the idea that the trypomastigotes

571   attachment to mammalian cells is an active process that requires energy and

572   mitochondrial respiratory chain activity as it was previously described (48).

573　　　　On the other hand, larger amounts of endogenous TcCox15 detected in the

574　　epimastigotes and amastigotes forms are consistent with the hypothesis that the

575　　replicative life-stages have a higher energy requirement than the infective stage. In

576　　consequence, the parasite would need a fully active respiratory chain, with a concomitant

577　　augmentation of heme A synthesis to assemble a higher amount of CcO complex. These

578　　concepts are supported by the over-expression of the recombinant non-functional

579　　TcCox15 that also impairs the intracellular replication of amastigotes. Likewise, this

580　　evidence confirms that heme A synthesis and CcO activity are essential for amastigote

581　　intracellular replication.

582　　　　In summary, we have demonstrated that TcCox15 plays the role of heme A

583　　synthase in *T. cruzi*. Despite the possible existence of a branched respiratory chain, our

584　　results clearly show that CcO type aa3 is the main terminal oxidase in all of *T. cruzi* life-

585　　cycle stages and that its activity is essential for parasite growth and infection. Remarkably,

586　　the data presented here demonstrate by the first time that impairment in CcO activity

587　　(inhibiting its heme A synthesis) negatively affects, not only the replicative stages, but the

588　　trypomastigotes infectivity. These evidences reinforce the idea of parasite energy

589　　metabolism as an attractive therapeutic target to control infection and transmission.

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597　　**DECLARATIONS OF INTEREST**

598　　The authors have declared that no competing interests exist.

599

600

606 **AUTHOR CONTRIBUTION STATEMENT**

607 JAC and MLM conceived, designed and supervised the project, MLM performed most of
608 designed experiments, BAC performed Western blot assays, SMM-B and JAC designed the
609 strategy to obtain the anti-TcCox15 antibodies. JAC, MLM, BAC and SMM-B discussed the
610 results. JAC and MLM wrote the manuscript with contributions from all other authors.

611

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736 mammalian cells requires parasite energy, and invasion can be independent of the
737 target cell cytoskeleton. Infect Immun. 1991;59(2):645–54.

738

739

740 **TABLE 1**

741

	heme A nmoles/ 1.10^9 cells ± S.D.	heme B nmoles/ 1.10^9 cells ± S.D.	heme A/heme B ± S.D.
vector	0.036 ± 0.005	1.225 ± 0.046	0.030 ± 0.003
TcCox15 + Tet	0.033 ± 0.003	1.477 ± 0.076	0.022 ± 0.001*
H307A + Tet	0.0160 ± 0.0003 *	1,207 ± 0.002	0.0136 ± 0.0003 **

742 **Table 1. The expression of recombinant TcCox15H307A.His enzyme decreases the**
743 **amount of heme A in *T. cruzi* epimastigotes.** Heme content of epimastigotes transfected
744 with pTcINDEX (vector), pTcINDEX.*TcCOX15.HIS* (TcCox15) or pTcINDEX.*TcCOX15H307A.HIS*
745 (H307A) treated with 0.15 μg/mL tetracycline for 3 days was quantified by heme pyridine
746 hemochrome method. The concentration of heme B and heme A were estimated from the
747 reduced minus oxidized spectra, using the molar extinction coefficient 23.98 mM^{-1} cm^{-1}
748 (557 nm) and 25.02 mM^{-1} cm^{-1} (588 nm) respectively (39). Data are expressed as means ±
749 S.D. of three technical replicates from one representative of at least three independent
750 experiments (biological replica). Asterisks indicate a statistically significant difference
751 compared to control, parasites transfected with pTcINDEX, *$P < 0.05$ and **$P < 0.01$ (one-
752 way analysis of variance followed by Tukey's Multiple Comparison post-test).

753

754

FIGURES LEGENDS

756 **Figure 1: Schematic representation of heme A synthesis**. Heme O Synthase (HOS) and
757 Heme A Synthase (HAS) (7).

758

759

760 **Figure 2. TcCox15 is detected in *T. cruzi* extracts from the different life-cycle stages.** (**A**)
761 Equal amounts of total cell-free extracts of epimastigotes (E), cell-derived trypomastigotes
762 (T), and amastigotes (A) were separated by SDS-PAGE, transferred to nitrocellulose, and
763 analyzed with anti-TcCox15, anti-tubulin as a loading control of total cell extract*,* and anti-
764 mitochondrial tryparedoxin peroxidase (anti-TcmPx) as a loading control as well as
765 mitochondrial marker. The Western blot is a representative from three independent
766 biological replicas. The relative amount of TcCox15 was quantified and normalized to anti-
767 tubuliun (**B**) and anti-TcmPx intensity (**C**). Data are expressed as means ± S.D. of three
768 independent assays. (**D**) Immufluorescence assay of *T. cruzi* epimastigotes transfected with
769 pTcINDEX.*TcCOX15.HIS* incubated with 0.15 µg/mL tetracycline for 3 days. TcCox15
770 (endogenous and recombinant proteins) was detected using anti-TcCox15 and FITC-
771 conjugated anti-rabbit (green) as secondary antibody. DNA was stained with DAPI (blue)
772 and mitochondrion was stained with Mitotracker (MT). DIC, differential interference
773 contrast image. The confocal images are a representative of multiple fields from three
774 different experiments. The colocalization analysis (Red:Green) was performed (Pearson's
775 correlation coefficient: 0.972, and Manders overlap coefficients: 0.895 and 0.924).

776

777

778 **Figure 3. The C-terminal GFP fusion to TcCox15 affects the heme A synthesis in yeast**
779 **cells.** (**A**) Spot growth assay of *cox15Δ* yeast cells transformed with p426.MET25 (vector),
780 p426.MET25.*TcCOX15.HIS* (TcCox15.His)*,* p426.MET25.*TcCOX15.HIS-GFP* (TcCox15.His-GFP)
781 or p426.MET25.*ScCOX15.HIS* (ScCox15). 5 µl of four serial dilutions from an initial cultures
782 of D^{600}=1 were plated on SC-URA Glc or SC-URA Gly-EtOH to test the respiratory capacity.

783   (**B**) The oxygen consumption rate of the same yeast cells was measured in 3% glycerol

784   (v/v). Data are expressed as means ± S.D. of three technical replicates from one

785   experiment representative of three independent biological replicas. (**C**) Western blot of

786   mitochondrial   extracts   from   *S.   cerevisiae   cox15Δ*   cells   transformed   with

787   p426.MET25.*TcCOX15.HIS* (TcCox15.His) or p426.MET25.*TcCOX15.HIS-GFP* (TcCox15.His-

788   GFP)*,* grown in SC-URA 0.2% Glucose, 1.8% Galactose, using the following antibodies: anti-

789   TcCox15, and anti-β subunit of F1 complex (anti-subβ F1) as a loading control of yeast

790   mitochondrial extract. (**D**) Confocal microscopy images of *S. cerevisiae cox15Δ* cells

791   transformed with p426.MET25.*TcCOX15.GFP* incubated overnight in SC-URA Gly-EtOH. (**E**)

792   Spot growth assay of WT yeast cells transformed with p426.MET25.*TcCOX15.HIS*

793   (TcCox15.His) or p426.MET25.*TcCOX15.HIS-GFP* (TcCox15.His-GFP). 5 µl of four serial

794   dilutions from an initial cultures of D^{600}=1 were plated in SC-URA Glc or Gly-EtOH

795   supplemented with 2mg/l or without (wo) methionine (increasing Met concentration

796   decrease MET25 promoter activity) to test the effect caused by increasing amount of

797   TcCox15.His-GFP on the respiratory capacity of the WT strain.

798

799

800   **Figure 4. The TcCox15 mutants do not exhibit HAS activity in *S. cerevisiae cox15Δ* cells.**

801   (**A**) Spot growth assay of *cox15Δ* yeast cells transformed with p426.MET25 (vector) or

802   *ScCOX15.HIS* (ScCox15), *TcCOX15.HIS* (TcCox15) and *TcCOX15HxxxA.HIS* mutants (H129A,

803   H206A and H307A). 5 µL of four serial dilutions from an initial culture of D^{600}=1 were

804   plated in solid SC-URA Glc or SC-URA Gly-EtOH and incubated during 5 days at 30 °C to test

805   their respiratory capacity. (**B**) The oxygen consumption rate of the same transformed yeast

806   cells previously grown overnight in SC-URA Glc was measured in 3% glycerol (v/v). Data are

807   expressed   as   means   ±   S.D.   of   three   technical   replicates   from   one   experiment

808   representative of three independent biological replicas. (**C**) Western blot of mitochondrial

809   extracts from the same transformed *cox15Δ* yeast cells*,* grown in SC-URA Glc.
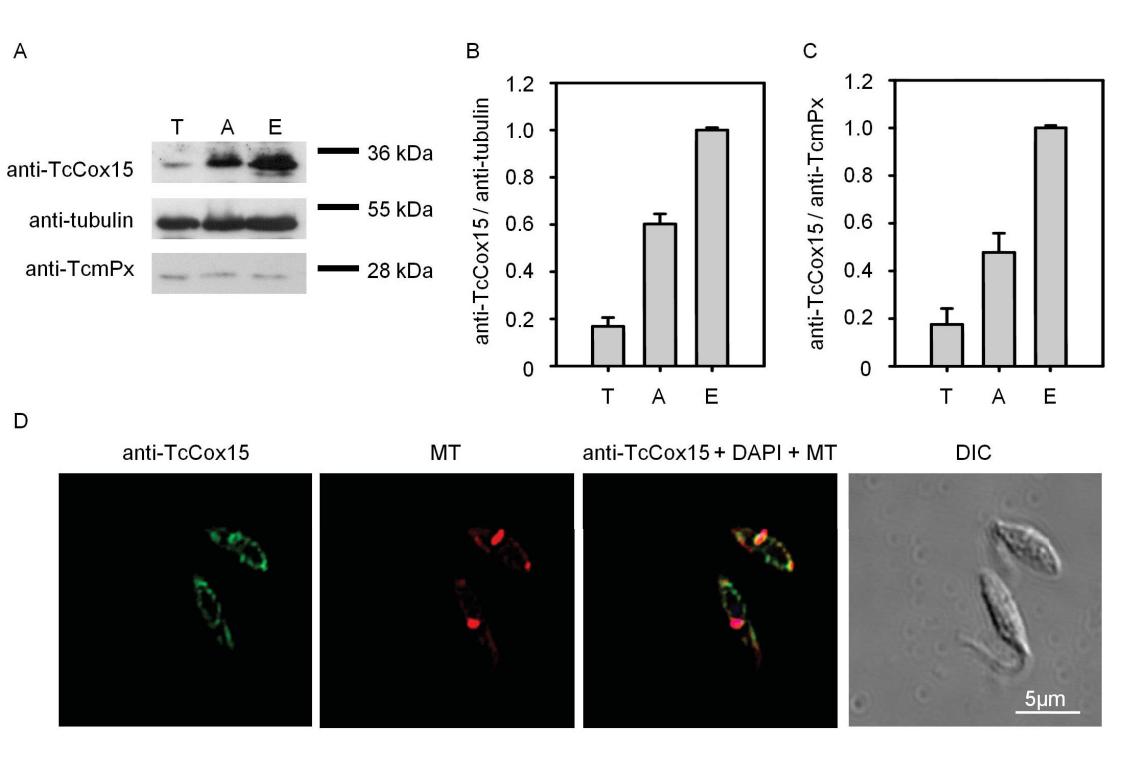
810

811

Figure 5. The recombinant TcCox15HxxxA mutants impair *T. cruzi* epimastigotes' growth. Growth curve of epimastigotes transfected with (**A**) pTcINDEX.*TcCOX15.HIS* (Tc15), (**B**) pTcINDEX.*TcCOX15H129A.HIS* (H129A), and (**C**) pTcINDEX.*TcCOX15H307A.HIS* (H307A), without or with the addition of 0.15 μg/ml, 0.25 μg/ml of tetracyclin (0, 0.15 Tet, 0.25 Tet). In all cases the growth of epimastigotes transfected with pTcINDEX (vector), incubated without and with 0.5 μg/ml of tetracycline, was monitored as control. Cells were maintained in mid-log phase by periodic dilutions every two days. Data are expressed as means ± S.D. of three independent biological replicas. (**D**) Quantification of the effect produced by the presence of recombinant proteins expressed as a percentage of parasite number of induced/non-induced conditions at day 8.

Figure 6. The recombinant TcCox15HxxxA mutants are detected in the epimastigotes' mitochondrion. (**A**) Western blot of total cell-free extracts from epimastigotes transfected with pTcINDEX (vector), pTcINDEX.*TcCOX15.HIS* (Tc15)*, pTcINDEX.*TcCOX15H129A.HIS* (H129A) and pTcINDEX.*TcCOX15H307A.HIS* (H307A) induced with a monodose of 0.05, 0.15, or 0.25 μg/ml tetracycline for 3 days, using the following antibodies: anti-TcCox15, anti-His, and anti-tubulin as a loading control. (**B**) Indirect immufluorescence assay of *T. cruzi* epimastigotes transfected with pTcINDEX (vector), pTcINDEX.TcCOX15H129A.HIS (H129A) or pTcINDEX.*TcCOX15H307A.HIS* (H307A), total TcCox15 was detected using anti-TcCox15 and FITC-conjugated anti-rabbit (green) as secondary antibody. DNA was stained with DAPI (blue) and mitochondrion with Mitotracker (MT). DIC, differential interference contrast image. The confocal images are a representative of multiple fields from three different experiments. The colocalization analysis (Red:Green) was performed on H129A (Pearson's correlation coefficient: 0.927, and Manders overlap coefficients: 0.895 and 0.924) and H307A (Pearson's correlation coefficient: 0.331, and Manders overlap coefficients: 0.717 and 0.908).

841   **Figure 7. The expression of the recombinant TcCox15H307A protein causes a reduction**

842   **on the oxygen consumption rate in *T. cruzi* epimastigotes.** The oxygen consumption rate

843   (nmoles of consumed O_2/min OD^{600}) of epimastigotes transfected with pTcINDEX (vector),

844   pTcINDEX.*TcCOX15.HIS* (Tc15) or pTcINDEX.*TcCOX15H307A.HIS* (H307A) was measured in

845   TSB-EGTA plus 5 mM succinic acid and normalized against parasite number. The

846   transfected epimastigotes were incubated without (non-induced) or with 0.25 µg/ml

847   tetracycline (induced) for 4 days. In each case the oxygen consumption rate was referred

848   to the non-induced condition. Data are expressed as means ± S.D. of three independent

849   biological replicas. Asterisk indicates a statistically significant difference of induced

850   compared to non-induced condition, *$P < 0.01$, each case was analyzed by two-tailed

851   paired Student's t test.

852

853

854   **Figure 8. The restriction in heme A synthesis (and C*c*O activity) impairs the**

855   **trypomastigotes' infection and intracellular amastigotes' replication.** To study the effect

856   of a restriction in heme A synthesis and CcO activity on *T. cruzi* infectivity and intracellular

857   replication, the capability of trypomastigotes to infect a cellular line, and intracellular

858   replication of amastigotes were evaluated in the presence of the recombinant

859   TcCox15H307A or TcCox15 proteins during the infection (+/-), post infection (-/+), in both

860   cases (+/+), and compared to control (-/-, without the expression of the recombinant

861   proteins). The infection and post infection incubation were performed in the absence or

862   presence of 0.20 µg/ml of tetracycline: -/- tetracycline never added to the medium; +/-

863   trypomastigotes were pre-treated with tetracycline for 1 h before the infection, it was

864   added only during the infection (16h) and then removed; -/+ trypomastigotes were not

865   induced, tetracycline was added only during 72h after the infection at the amastigote

866   stage; +/+, trypomastigotes were pre-treated and tetracycline was maintained during the

867   whole assay. The cells were stained with Giemsa reagent and the percentage of infected

868   cell (infection capacity) and number of amastigotes per infected cell (intracellular

869   replication) evaluated under light microspcope. (**A** and **D**) The percentage of infected cells

870   is expressed as means ± S.D. of three independent biological replicates and analyzed by

One-way analysis of variance followed by Dunnett's Multiple Comparison post-test (*$P <$ 0.001). (**B** and **E**) The number of intracellular amastigotes per infected cell is shown in a box and whisker plot (the box indicated the first quartil, the median and the third quartil, and the whisker indicated the 5th and 95th percentile), the mean is indicated with a dash, and analyzed by Kruskal-Wallis test followed by Dunn's Multiple Comparison post-test (**$P < 0.001$). (**C** and **F**) Images of Giemsa-satined infected cells. (**G**) Western blot of total cell-free extracts from cell-derived trypomastigotes (T) and amastigotes (A) transfected with pTcINDEX.*TcCOX15.HIS* (Tc15) or pTcINDEX.*TcCOX15H307A.HIS* (H307A) treated with 0.20 µg/ml of tetracycline for 0, 1 and 16 h (T) or during 0 and 48h (A), using the following antibodies: anti-TcCox15 and anti-tubulin as a loading control.

Heme B   **HOS**   Heme O   **HAS**   Heme A

A

	T	A	E	
anti-TcCox15				36 kDa
anti-tubulin				55 kDa
anti-TcmPx				28 kDa

B

anti-TcCox15 / anti-tubulin

C

anti-TcCox15 / anti-TcmPx

D

anti-TcCox15   MT   anti-TcCox15 + DAPI + MT   DIC

5μm

A

vector
TcCox15.His
TcCox15.His-GFP
ScCox15.His

Glc

vector
TcCox15.His
TcCox15.His-GFP
ScCox15.His

Gly-EtOH

B

C

anti-TcCox15

72 kDa
55 kDa
36 kDa
28 kDa

anti-subβ F1

anti-GFP

72 kDa
55 kDa
36 kDa
28 kDa

anti-subβ F1

D

5μm

E

TcCox15.His
TcCox15.His-GFP

Glc

Gly-EtOH
2 mg/l Met

Gly-EtOH
wo Met

A

Vector
ScCOX15
TcCOX15
H129A
H206A
H307A

Glc   Gly-EtOH

B

H307A   H206A   H129A   TcCOX15

anti-
TcCox15

C

vector   H307A   H206A   H129A   TcCOX15

anti-
TcCox15   36 kDa

	% of growth Induced/Non-induced
Tc15 + 0.15 Tet	76.2
Tc15 + 0.25 Tet	42.8
H129A + 0.15 Tet	22.1
H129A + 0.25 Tet	7.7
H307A + 0.15 Tet	18.5
H307A + 0.25 Tet	6.1

A

[Tet] (µg/mL)	0 - vector	0	0.05	0.15	0.25	0.5 - vector	0	0.05	0.15	0.25	0.5 - vector	0	0.05	0.15	0.25

Tc15 / H129A / H307A

anti-TcCox15
anti-His
anti-tubulin

B

	anti-TcCox15	MT	anti-TcCox15 + DAPI + MT	DIC
vector				5µm
H129A				5µm
H307A				5µm