



# A new model for *Trypanosoma cruzi* heme homeostasis depends on modulation of TcHTE protein expression

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Heme is an essential cofactor for many biological processes in aerobic organisms, which can synthesize it *de novo* through a conserved pathway. *Trypanosoma cruzi*, the etiological agent of Chagas disease, as well as other trypanosomatids relevant to human health, are heme auxotrophs, meaning they must import it from their mammalian hosts or insect vectors. However, how these species import and regulate heme levels is not fully defined yet. It is known that the membrane protein TcHTE is involved in *T. cruzi* heme transport, although its specific role remains unclear. In the present work, we studied endogenous TcHTE in the different life cycle stages of the parasite to gain insight into its function in heme transport and homeostasis. We have confirmed that TcHTE is predominantly detected in replicative stages (epimastigote and amastigote), in which heme transport activity was previously validated. We also showed that in epimastigotes, TcHTE protein and mRNA levels decrease in response to increments in heme concentration, confirming it as a member of the heme response gene family. Finally, we demonstrated that *T. cruzi* epimastigotes can sense intracellular heme by an unknown mechanism and regulate heme transport to adapt to changing conditions. Based on these results, we propose a model in which *T. cruzi* senses intracellular heme and regulates heme transport activity by adjusting the expression of TcHTE. The elucidation and characterization of heme transport and homeostasis will contribute to a better understanding of a critical pathway for *T. cruzi* biology allowing the identification of novel and essential proteins.

*Trypanosoma cruzi* is a protozoan parasite responsible for Chagas disease, the most prevalent parasitic disease in several countries of the Americas. It is a trypanosomatid, member of the class Kinetoplasteae, that also includes other parasites affecting human health such as *Trypanosoma brucei* (sleeping sickness) and *Leishmania* spp. (visceral, cutaneous, and mucocutaneous leishmaniasis). *T. cruzi* undergoes a complex life cycle, alternating between a mammal host and an insect vector

and displaying at least four life cycle stages that are morphologically and metabolically different: Epimastigotes, metacyclic trypomastigotes, intracellular amastigotes, and bloodstream trypomastigotes (1). Trypanosomatids are aerobic organisms that present several heme proteins involved in essential metabolic pathways (2). However, they lack a complete heme synthesis pathway (3). For this reason, trypanosomatids must scavenge this molecule from the host or vector (2). Also, it is well-established that free heme is not found in any living cell because of its toxicity; therefore, a fine-tuned control of heme homeostasis is necessary to avoid its harmful effects.

In the last years, some proteins of trypanosomatids displaying sequence homology to CeHRG-4 (*Caenorhabditis elegans* heme responsive gene 4) (4) have been studied and proposed as membrane transporters. LHR1 (*Leishmania* heme response 1, from *Leishmania amazonensis*) was the first protein postulated as a heme transporter in *Leishmania* spp. LHR1 mRNA responds to heme availability in the environment, confirming it as a member of the heme response gene (HRG) family (5). Also, heme uptake mediated by LHR1 results are relevant for the virulence of *L. amazonensis* (6, 7). We described previously a homologous protein in *T. cruzi* named TcHTE (*T. cruzi* heme transport enhancer) that plays a critical role in heme transport and is found mainly in the flagellar pocket of *T. cruzi* epimastigotes (8). TbHRG (*T. brucei* heme responsive gene) was described in *T. brucei* and postulated to be involved in transport of free heme or in the salvage of heme derived from hemoglobin degradation (9, 10). More recently, the protein LmFLVCRb, member of the major facilitator superfamily, was described as a heme importer in *Leishmania major* (11). The latest might be involved in free heme transport in *L. major*, probably overlapping some functions with LmHR1.

LHR1, TcHTE, and TbHRG present similar growth performance when expressed in *Saccharomyces cerevisiae* *hem1Δ*, allowing these knockout cells to grow in a medium supplemented with low heme. Also, the overexpression of recombinant versions of these trypanosomatid genes in the corresponding native organisms causes an increment in the intracellular heme concentration, confirming their role in heme transport (5, 8, 9). Bioinformatic analysis of these proteins predict that they have a four transmembrane segment topology similar to CeHRG-4 (4, 5, 8, 9). However, it remains unclear how the HRG proteins work, which are their precise roles in heme transport, and how the expression of HRG genes is regulated by heme.

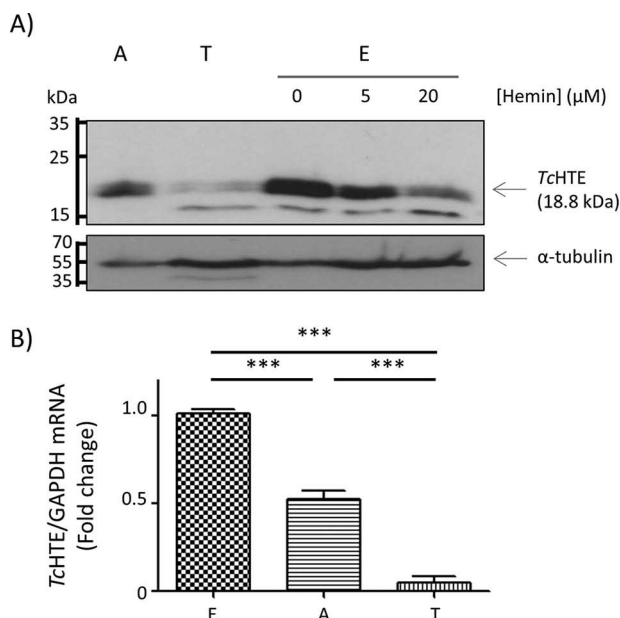
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**Figure 1. TcHTE is up-regulated in the replicative life cycle stages.** A, Western blot analysis using anti-TcHTE antibodies to detect the endogenous expression of TcHTE in different life cycle stage of *T. cruzi*: Intracellular amastigotes (A), trypomastigotes (T), and epimastigotes (E). The epimastigotes were incubated in axenic culture for 3 days in LIT–10% FBS supplemented without or with 5 and 20  $\mu\text{M}$  hemin (0, 5, and 20). Anti-tubulin was used as a loading control. B, qRT-PCR analysis to detect TcHTE transcript level in epimastigotes from axenic culture (E), intracellular amastigotes (A), and trypomastigotes (T) of *T. cruzi*. Transcripts from intracellular amastigotes and cell-derived trypomastigotes were compared with epimastigotes grown in LIT–10% FBS plus 5  $\mu\text{M}$  hemin, which was set to 1. GAPDH was used for normalization. Data are presented as mean  $\pm$  S.D. of three independent assays. Statistical significance was determined by one-way ANOVA followed by Bonferroni’s multiple comparisons test (\*\*\*,  $p < 0.001$ ).

In this work, we present the study of the endogenous TcHTE. Our data clearly show that TcHTE transcription is regulated by heme as LHR1 does (5). TcHTE mRNA and protein levels decrease in response to increments in heme concentration, which confirms TcHTE as a member of the HRG family. Using different fluorescent heme analogs, we proved that epimastigotes sense intracellular heme concentration and consequently modulate the amount of TcHTE. Besides, the expression of recombinant TcHTE enhances replication of intracellular amastigotes, probably by increasing heme uptake from the cytoplasm of the infected cell, where its availability could be a limiting growth factor. In summary, our results show that heme transport activity in *T. cruzi* is tightly modulated by the presence or absence of TcHTE.

## Results

### The accumulation of TcHTE (mRNA and protein) changes through the different life cycle stages of *Trypanosoma cruzi*

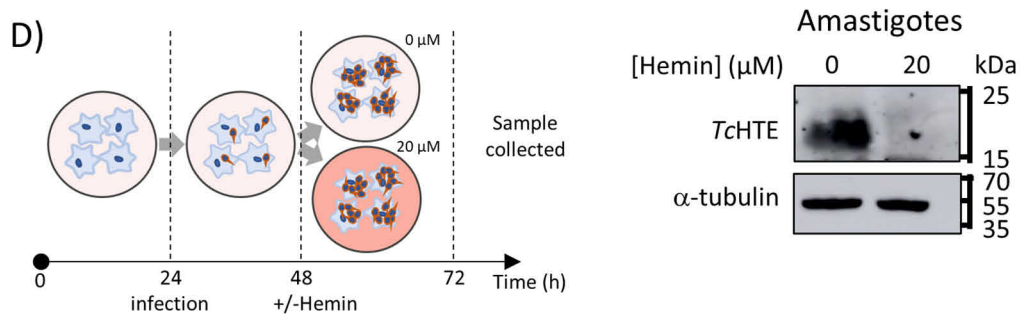
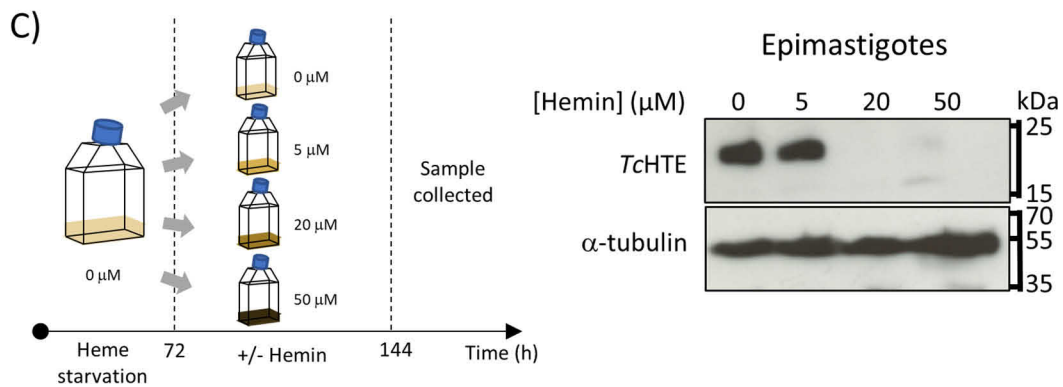
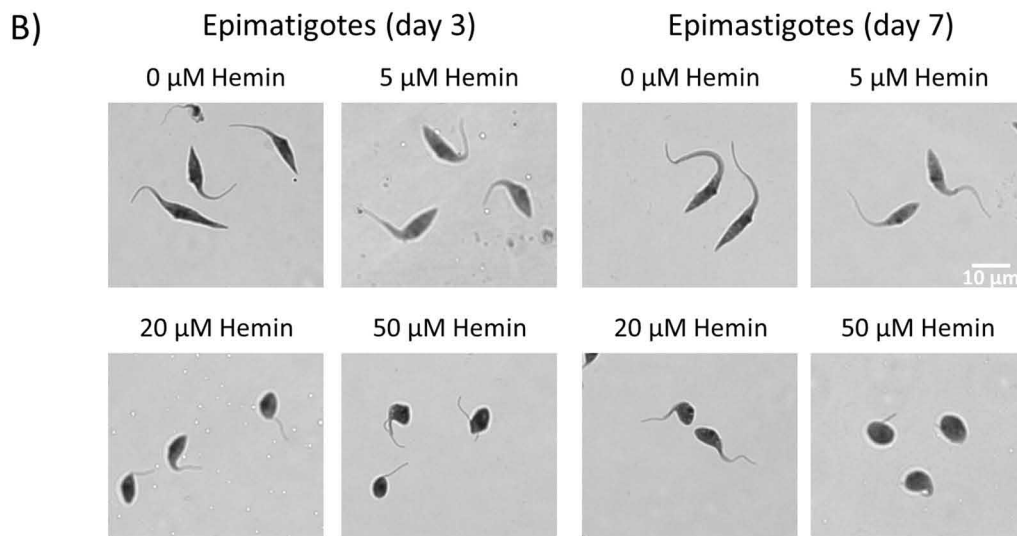
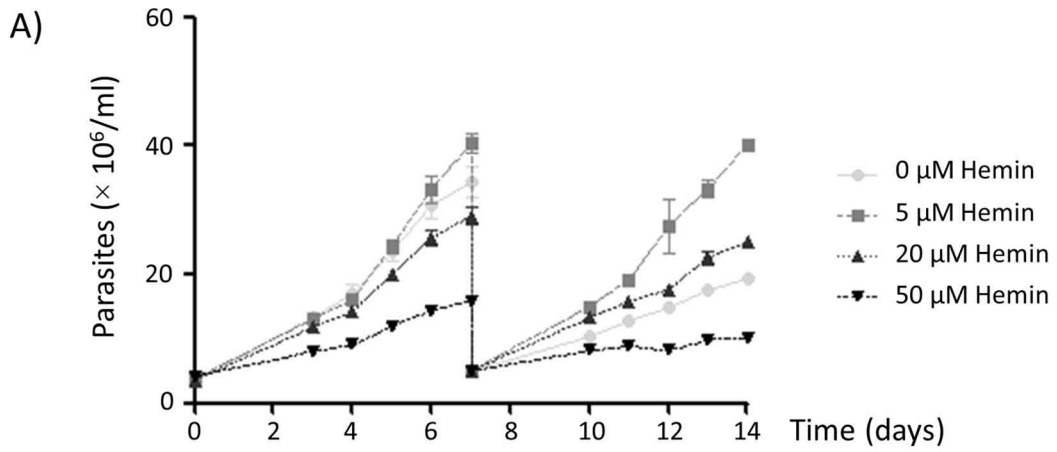
TcHTE was described previously as a critical protein for heme transport in *T. cruzi*, probably being part of the heme transporter and/or regulating its activity (8). To expand our knowledge of TcHTE, we used specific polyclonal antibodies against TcHTE to detect and analyze the presence of the endogenous protein along the *T. cruzi* life cycle stages. The Western blotting assays revealed that TcHTE was highly expressed in amastigotes and epimastigotes (replicative stages) and almost

undetectable in trypomastigotes (the infective and nonreplicative stage), as it is shown in Fig. 1A. TcHTE expression varied together with hemin concentration in the medium when epimastigotes were incubated 3 days in LIT–10% FBS with 0, 5, and 20  $\mu\text{M}$  hemin. The signal corresponding to this protein was more intense at lower hemin concentrations. The lower molecular weight bands observed in this blot could be product of protein degradation during this sample preparation. Also, we analyzed the accumulation level of TcHTE mRNA by qRT-PCR assays. The amount of TcHTE mRNA was significantly higher in epimastigotes than amastigotes. Also, the amounts detected in epimastigotes and amastigotes were significantly higher compared with trypomastigotes, as it is shown in Fig. 1B. These results indicate that TcHTE was up-regulated at mRNA and protein level in the replicative stages compared with the infective stage.

### Variation in the concentration of hemin affects the amount of TcHTE detected in replicative life cycle stages

To explore how changes in the amount of hemin added to the culture medium modulate the amount of TcHTE present in cells, we first reviewed the growth conditions reported for epimastigotes. Previously, we have shown that *T. cruzi* incorporates heme and heme analogues (HAs) in the replicative stages to fulfill the requirements for this cofactor. The addition of 5–20  $\mu\text{M}$  hemin to the medium did not affect epimastigotes’ growth and, in stationary state, the intracellular heme concentration was approximately the same in both cases. However, higher concentrations of hemin produced a growth defect, being highly toxic at 100  $\mu\text{M}$  (8). Then, we first analyzed the effect caused by an increment in hemin concentration in the medium but below lethal concentrations (50  $\mu\text{M}$  or less). Epimastigotes routinely maintained in a medium with 5  $\mu\text{M}$  hemin were collected, washed, and starved for heme for 72 h (medium without hemin). After that, parasites were cultured with 0, 5, 20, or 50  $\mu\text{M}$  hemin for 7 days. Then, cultures were diluted in fresh media (maintaining the same hemin concentration) and the growth was followed until day 14 (Fig. 2A). At day 7, slight differences in the total parasite number were observed between epimastigotes grown with 0, 5, and 20  $\mu\text{M}$  hemin and also a moderate negative effect at 50  $\mu\text{M}$  hemin. However, during the second week, the negative effect produced by the absence (no hemin added) and higher hemin concentrations (20 and 50  $\mu\text{M}$ ) was more severe. The addition of 5  $\mu\text{M}$  hemin resulted the optimal concentration to allow parasite growth in our laboratory conditions. Also, on the third and seventh days of this experiment, samples were collected, stained with Giemsa reagent, and analyzed by optical microscopy (Fig. 2B). The epimastigotes challenged to grow with 0 or 5  $\mu\text{M}$  hemin presented a conserved elongated shape, as typically described for this stage. However, those maintained with 20 or 50  $\mu\text{M}$  hemin presented a rounded shape, possibly as a consequence of a potential toxic effect caused by the amount of hemin present in these media (12). TcHTE protein accumulation was analyzed by Western blotting assays after 72 h of incubation in the mentioned conditions (Fig. 2C, left panel shows incubations scheme). The protein signal was observed in samples taken from 0 and 5  $\mu\text{M}$

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hemin conditions, but it was undetectable in 20 or 50  $\mu\text{M}$  hemin, as it is shown in Fig. 2C (right panel), in agreement with results reported in Fig. 1A.

Analyzing the effect of heme on TcHTE expression in intracellular amastigotes presents a difficulty, because the addition of hemin to the medium during the whole assay (infection and replication) results in cell toxicity. Then, the accumulation of TcHTE in this stage was evaluated in samples taken from infected cells that were treated without (as control) or with 20  $\mu\text{M}$  hemin for only 24 h (Fig. 2D, left panel shows the scheme of treatment). The amastigotes were purified and the presence of TcHTE was analyzed by Western blotting. The signal corresponding to TcHTE was clearly detected in the samples corresponding to amastigote control, but the addition of 20  $\mu\text{M}$  hemin caused a significant reduction in the amount of TcHTE, being almost undetectable, as is shown in the right panel of Fig. 2D. In summary, TcHTE was detected in the replicative forms of *T. cruzi*, and it was more abundant under heme deprivation or low heme availability.

#### The amount of TcHTE (mRNA and protein) detected in epimastigotes responds inversely to changes in the concentration of hemin

To investigate how TcHTE expression responds to hemin, we analyzed the accumulation of TcHTE mRNA when epimastigotes were challenged to a medium containing lower or higher concentration of hemin. Briefly, epimastigotes preincubated with 0 or 20  $\mu\text{M}$  hemin for 72 h were collected, washed, and incubated with 0, 5, and 20  $\mu\text{M}$  hemin for 18 h. In both cases, changes in the amount of TcHTE mRNA relative to GAPDH (housekeeping gene) were quantified by qRT-PCR and results are presented in Fig. 3A. When epimastigotes preincubated with 20  $\mu\text{M}$  hemin were changed to a medium without hemin (0  $\mu\text{M}$ ), the amount of TcHTE mRNA significantly increased, at least three times (Fig. 3A, right panel). On the other hand, when epimastigotes preincubated without hemin were changed to 5 or 20  $\mu\text{M}$  hemin, the amount of accumulated TcHTE mRNA significantly decreased in both cases, about two and three times, respectively (Fig. 3A, left panel). These results confirmed TcHTE as a member of HRG family because mRNA accumulation was regulated by heme. (Fig. S2, phylogenetic tree of representative HRGs proteins).

Additionally, changes in TcHTE protein expression were evaluated by Western blot assays. Briefly, epimastigotes routinely maintained in medium with 5  $\mu\text{M}$  hemin were transferred to 30  $\mu\text{M}$  hemin for 7 days. On day 8, epimastigotes were washed and transferred to fresh medium without hemin (0  $\mu\text{M}$ ) for 7 days (left panel of Fig. 3B shows the scheme of this assay). Samples were taken every day along the whole assay. The results, presented in Fig. 3B (right panel), show that the protein

signal almost disappeared 24 h after epimastigotes were incubated with 30  $\mu\text{M}$  hemin. On the other hand, when epimastigotes were transferred to a hemin-free medium, 48 h were required to restore TcHTE signal. To follow the drop of TcHTE during the first day of increasing hemin, epimastigotes maintained with 5  $\mu\text{M}$  were transferred to 30  $\mu\text{M}$  hemin, samples were taken every hour and treated for Western blot assays. Fig. 3C clearly shows that TcHTE signal became almost undetectable 15–17 h after hemin concentration was increased.

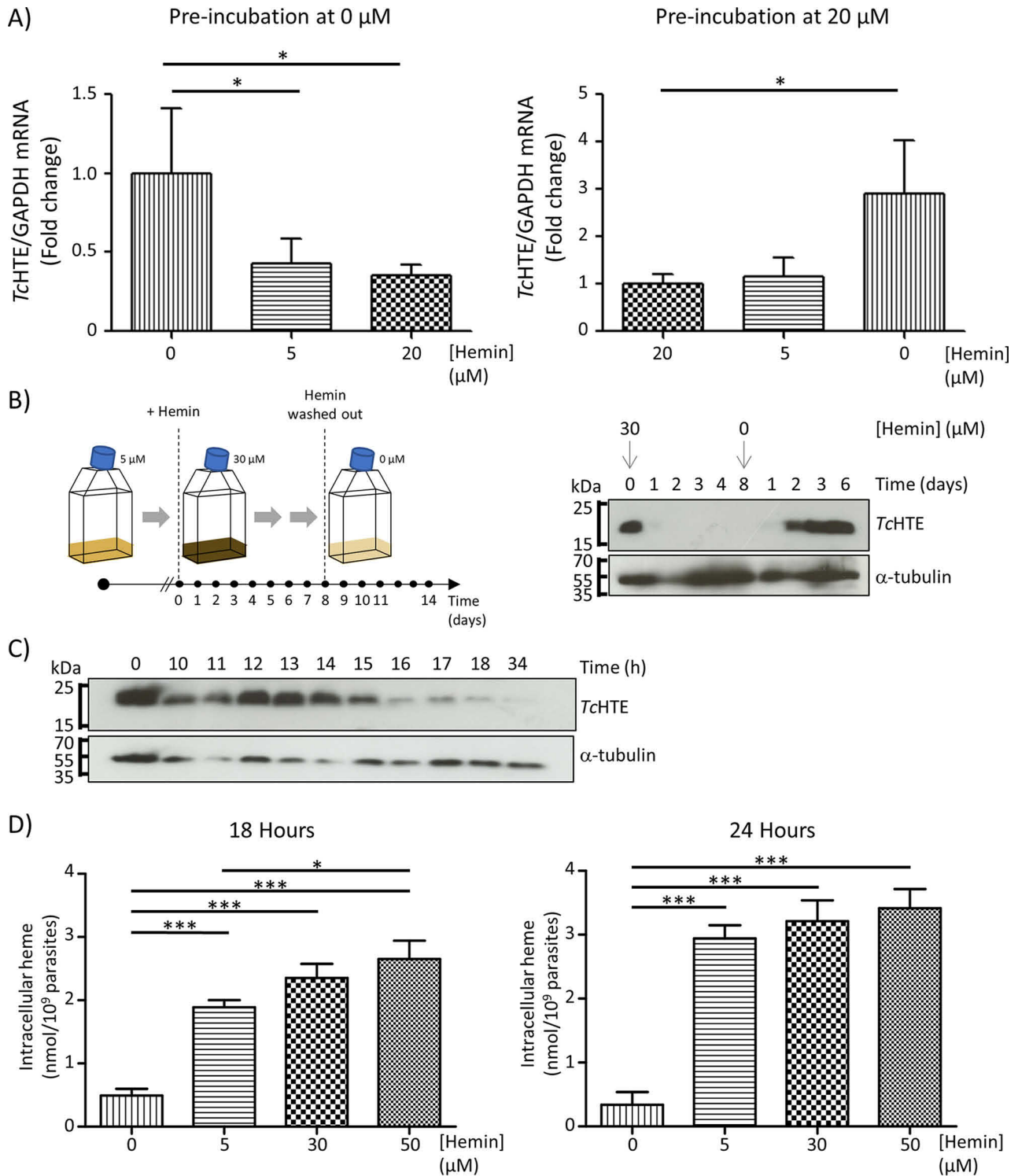
Furthermore, we quantified intracellular heme in epimastigotes (which were previously starved for heme for 48 h) after 18 and 24 h of incubation with 0, 5, 30, and 50  $\mu\text{M}$  hemin. Intracellular heme significantly increased in samples incubated with hemin (18 and 24 h) compared with the sample maintained without hemin (Fig. 3D, both panels). Also, analysis of samples taken at 18 h of incubation showed a significant difference in intracellular heme of epimastigotes incubated with 5 and 50  $\mu\text{M}$  hemin (Fig. 3D, left panel). This difference disappeared after 24 h of incubation (Fig. 3D, right panel) and intracellular heme reached almost the same value of  $\sim 3$  nmol/ $10^9$  parasites.

#### Epimastigotes can sense intracellular heme (and HAs) to modulate TcHTE protein level

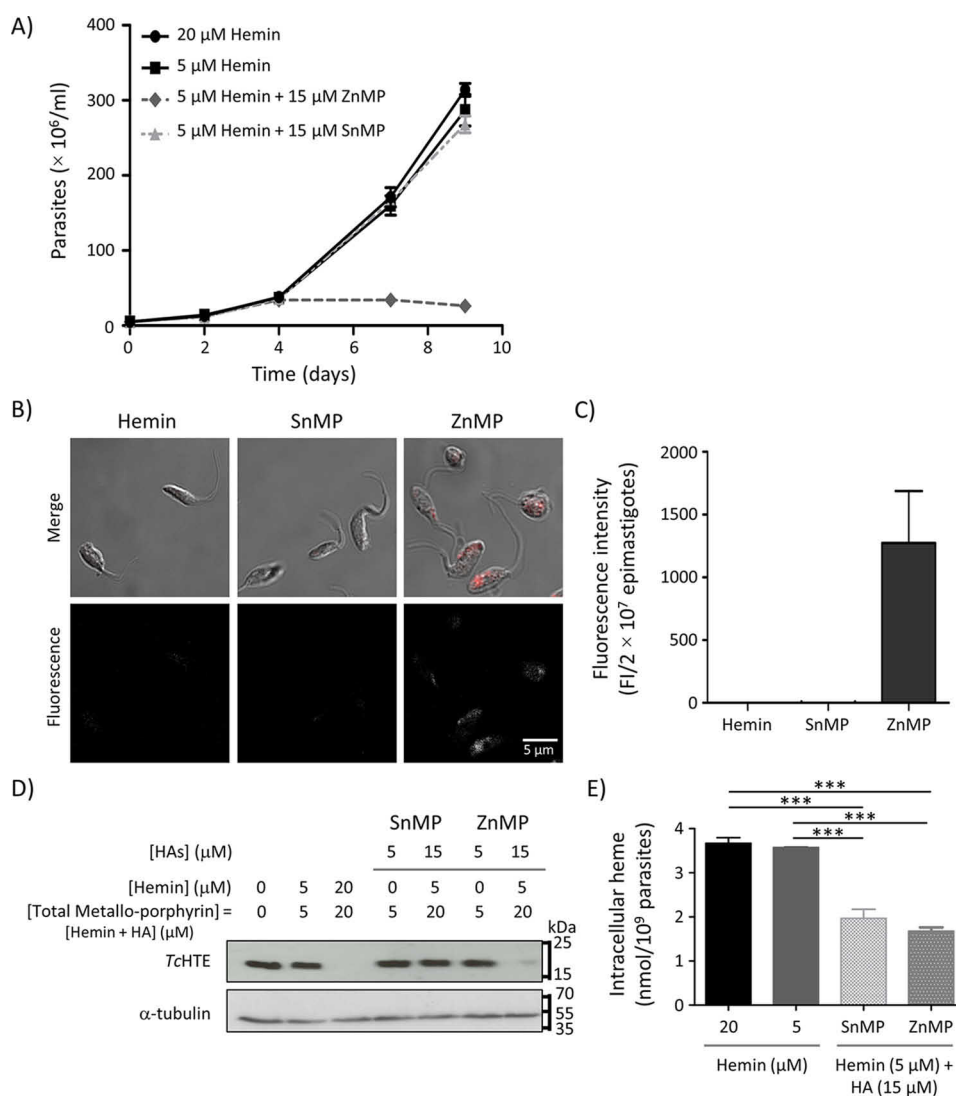
We have previously shown that fluorescent HAs that have been used to evaluate heme transport activity in *T. cruzi* (8, 13, 14), can be selectively incorporated by the replicative stages of the parasite (8). Based on this potentiality, we designed a strategy to evaluate if epimastigotes sense intracellular or environmental heme (or HAs) to modulate TcHTE expression. First, epimastigotes were challenged to grow under nontoxic concentration of “total metalloporphyrins” of 20  $\mu\text{M}$  or lower (total metalloporphyrins is referred to hemin plus different HAs), and parasite proliferation and HAs internalization were analyzed. The growth profile of epimastigotes in a medium containing hemin (5 or 20  $\mu\text{M}$ ) or 5  $\mu\text{M}$  hemin plus 15  $\mu\text{M}$  HA (20  $\mu\text{M}$  of total metalloporphyrins) is shown in Fig. 4A. Epimastigotes incubated with 5 or 20  $\mu\text{M}$  hemin or with 5  $\mu\text{M}$  hemin plus 15  $\mu\text{M}$  SnMP (Sn(IV) mesoporphyrin IX) did not show any significant difference in the growth profile. On the other hand, parasites incubated with 5  $\mu\text{M}$  hemin plus 15  $\mu\text{M}$  ZnMP (Zn(II) mesoporphyrin IX) were severely affected, presumably because of ZnMP toxicity. Samples were taken at the third day of the assay and the presence of HAs inside cells was analyzed by confocal microscopy and direct fluorescent measurements as we reported previously for heme/HAs transport activity (8). Fig. 4, B and C, show that epimastigotes incubated with SnMP (5  $\mu\text{M}$  hemin plus 15  $\mu\text{M}$  SnMP) did not exhibit any significant fluorescent signal detected by confocal microscopy or direct measurements of fluorescence, suggesting that it was not imported

**Figure 2. Changes in heme concentration in the medium affects epimastigote proliferation and TcHTE expression.** A, growth curves of epimastigotes in LIT–10% FBS plus 0, 5, 20, and 50  $\mu\text{M}$  hemin, with a dilution to the initial concentration in fresh medium at day 7. Previously, parasites were maintained 72 h in LIT–10% FBS without hemin. Data are presented as mean  $\pm$  S.D. of three independent assays. B, Giemsa staining of epimastigotes cultured in LIT–10% FBS with 0, 5, 20, or 50  $\mu\text{M}$  hemin for 3 and 7 days. C, scheme of the experimental design to analyze endogenous TcHTE expression in epimastigotes (left panel). Western blot analysis (right panel) using anti-TcHTE antibodies shows the accumulation of TcHTE protein in total extracts of epimastigotes incubated for 72 h without or with low hemin. Anti-tubulin was used as a loading control. D, scheme of the experimental design to analyze endogenous TcHTE expression in amastigotes (left panel). Western blot analysis (right panel) using anti-TcHTE antibodies shows the accumulation of TcHTE protein in total extracts of amastigotes incubated for 24 h without or with 20  $\mu\text{M}$  hemin. Anti-tubulin was used as a loading control.

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**Figure 3. TcHTE expression responds to heme at mRNA and protein level in epimastigotes.** A, quantification of TcHTE mRNA level in epimastigote with different concentrations of hemin. Epimastigotes were cultured in LIT-10% FBS without (left panel) or with 20  $\mu\text{M}$  hemin (right panel). 3 days after, cells were collected, washed with PBS, and cultured in LIT-10% FBS supplemented with 0, 5, or 20  $\mu\text{M}$  heme for 18 h and TcHTE mRNA was quantified by qRT-PCR. GAPDH was used for normalization. Data are presented as mean  $\pm$  S.D. of three independent assays. Statistical significance was determined by one-way ANOVA followed by Bonferroni's multiple comparisons test (\*,  $p < 0.05$ ). B, quantification of TcHTE protein level in epimastigote with different concentration of hemin. Scheme of the experimental design to analyze endogenous TcHTE protein expression in epimastigote stage. Parasites were grown in LIT-10% FBS plus 5  $\mu\text{M}$  hemin and then incubated in LIT-10% FBS plus 30  $\mu\text{M}$  hemin (day 0). Samples were taken every day for a week. The remaining epimastigotes were washed and maintained in LIT-10% FBS without hemin (day 8) and samples were taken every day for another week (left panel). Western blot assay (right panel) using anti-TcHTE antibodies to recognize endogenous protein in total extracts of epimastigotes. Anti-tubulin was used as loading control. C, epimastigotes grown in LIT-10% FBS supplemented with 5  $\mu\text{M}$  hemin were then incubated in LIT-10% FBS plus 30  $\mu\text{M}$  hemin. Samples were taken every hour. Western blot assay used anti-TcHTE antibodies to recognize endogenous protein in total extracts of epimastigotes. Anti-tubulin was used as loading control. D, intracellular heme content determined by pyridine method in epimastigotes grown in LIT-10% FBS plus 0, 5, 30, and 50  $\mu\text{M}$  hemin for 18 h (left panel) and 24 h (right panel). Data are presented as mean  $\pm$  S.D. of three independent assays. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ).



**Figure 4. Heme analogs affect epimastigote growth and TcHTE accumulation.** A, growth curves of *T. cruzi* epimastigotes in LIT-10% FBS plus 5, 20  $\mu$ M hemin or 5  $\mu$ M hemin plus 15  $\mu$ M of HAs (ZnMP and SnMP), monitored for 9 days. Parasites were maintained in exponential growth by periodic dilution every 2 days in fresh medium. Previously, parasites were maintained 72 h in LIT-10% FBS without hemin. Data are presented as mean  $\pm$  S.D. of three independent assays. B, confocal microscopy of epimastigotes incubated with 5  $\mu$ M hemin or 5  $\mu$ M hemin plus 15  $\mu$ M different HAs for 3 days. C, fluorescence intensity from cell-free extracts were obtained after 3 days of incubation of epimastigotes with 5  $\mu$ M hemin or 5  $\mu$ M hemin plus 15  $\mu$ M different HAs. The experimental data are presented as the mean  $\pm$  S.D. of three independent replicates. D, Western blot assay, using anti-TcHTE antibodies to recognize endogenous protein in total extracts of epimastigotes cultured with 0, 5, or 20  $\mu$ M hemin, 5  $\mu$ M HAs, or 5  $\mu$ M hemin plus 15  $\mu$ M HAs for 4 days. Anti-tubulin was used as a loading control. E, intracellular heme content determined by pyridine method of epimastigotes grown in LIT-10% FBS plus 5, 20  $\mu$ M hemin, or 5  $\mu$ M hemin plus 15  $\mu$ M HAs, for 72 h. Data are presented as mean  $\pm$  S.D. of three independent assays. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test (\*\*\*,  $p < 0.001$ ).

or it was rapidly exported. In any case, SnMP did not remain inside the cell. On the other hand, epimastigotes incubated with ZnMP (5  $\mu$ M hemin plus 15  $\mu$ M ZnMP) showed an intense fluorescence signal, detected by confocal microscopy and direct fluorescence measurements, confirming its incorporation by the cell. These observations are in agreement with previous results in which we have shown that ZnMP, but not SnMP, is internalized by *T. cruzi* (8). The effect of these HAs on TcHTE accumulation was analyzed by Western blot assay in samples taken after 3 days of incubation with the HAs (0, 5, 20  $\mu$ M hemin; 5  $\mu$ M HAs; or 5  $\mu$ M hemin plus 15  $\mu$ M HAs) and the results are shown in Fig. 4D. The signal corresponding to TcHTE was detected in samples from 0 and 5  $\mu$ M hemin, 5  $\mu$ M of SnMP, 5  $\mu$ M of hemin plus 15  $\mu$ M of SnMP (20  $\mu$ M total metalloporphyrins), and with 5  $\mu$ M of ZnMP conditions. On the other hand,

TcHTE was not detected in samples from either 5  $\mu$ M of hemin plus 15  $\mu$ M of ZnMP or 20  $\mu$ M of hemin. These results indicate that only those HAs that were internalized and remained inside the parasite provoked a reduction on TcHTE protein level similar to the reduction produced by higher hemin.

When we analyzed intracellular heme, we observed that the treatment with both HAs (ZnMP and SnMP) significantly reduced intracellular heme content, indicating that somehow SnMP impaired heme uptake, causing a drop in intracellular heme levels, as is shown in Fig. 4E.

#### rTcHTE.His-GFP enhances replication of amastigotes

The replication of intracellular amastigotes was analyzed in WT and recombinant parasites overexpressing rTcHTE.His-GFP. Briefly, monolayers of Vero cells were infected with WT

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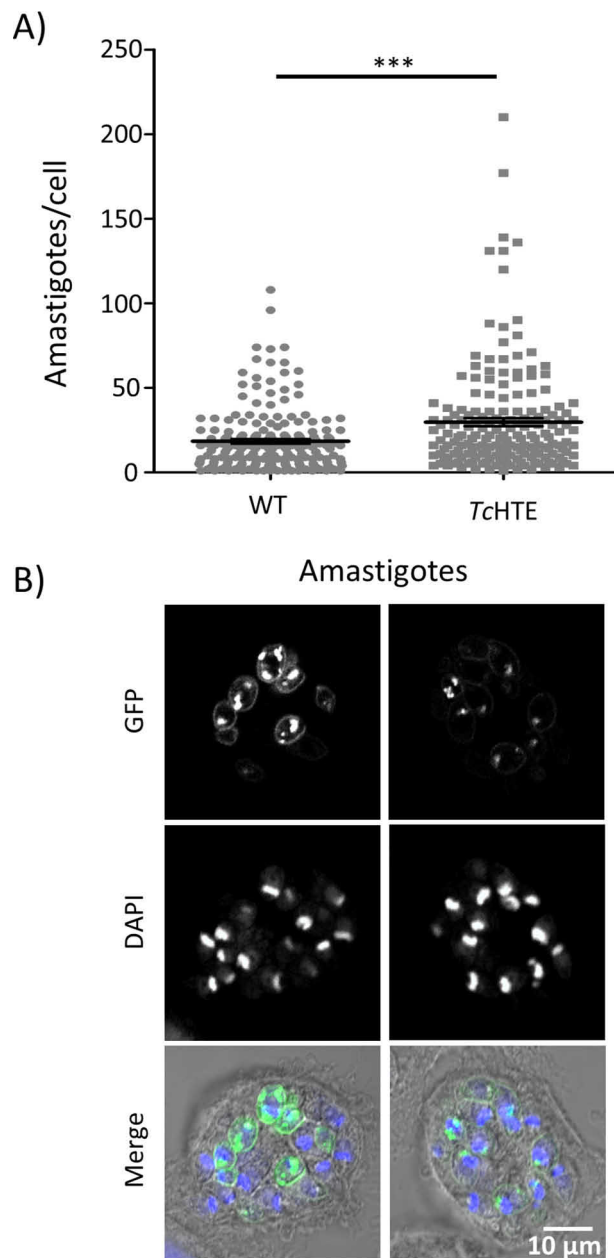
or rTcHTE.His-GFP overexpressing trypomastigotes. 48 h post infection, cells were fixed, stained with Giemsa reagent, and amastigotes were counted (Fig. 5A). The number of intracellular amastigotes was significantly higher in cells infected with rTcHTE.His-GFP overexpressing parasites compared with those infected with WT parasites. ( $29 \pm 2$  and  $18 \pm 1$  amastigotes/infected-cell, respectively,  $p < 0.001$ ). This result suggests that the overexpression of TcHTE increased amastigote replication rate in a low free-heme environment as the cellular cytosol.

Additionally, we also analyzed the presence of rTcHTE.His-GFP in intracellular amastigotes. The images recorded for intracellular amastigotes confirmed rTcHTE.His-GFP expression and showed that its signal is highly intense and punctuated in a region over the plasma membrane that could overlap with the flagellar pocket region (Fig. 5B), as was previously shown in epimastigotes (8). However, several amastigotes also showed a fluorescent signal distributed along the plasma membrane. Unfortunately, endogenous TcHTE could not be detected by indirect immunofluorescence assays, using anti-TcHTE antibodies. Then, the difference observed in the localization of rTcHTE.His-GFP protein in amastigotes could be an artifact of the protein overexpression because of the use of nonclonal recombinant parasites or could indicate that TcHTE presents different localization in the intracellular stage.

### Discussion

In this work we present data that clarify the relationship between TcHTE and heme transport activity in *T. cruzi*. The attainment of polyclonal antibodies against TcHTE allowed the evaluation of the endogenous protein. It was clearly detected in epimastigotes and amastigotes, and almost undetectable in trypomastigotes. In accordance, quantitative RT-PCR analysis showed that TcHTE mRNA was significantly higher in the replicative life cycle stages of *T. cruzi*. TcHTE (as mRNA and protein) was more abundant in the stages in which *T. cruzi* is able to import heme (or HAs) (8). In addition, the amount of hemin added to the medium inversely affected the amount of TcHTE mRNA detected in epimastigotes, as it was reported for LHR1 promastigotes (5). These results confirmed TcHTE as a member of the HRG family (4). It is important to note that *Leishmania* (5) and *T. cruzi* HRGs respond to heme but not TbHRG (10), suggesting that those gene products might be under different regulations and/or play different roles in heme transport.

Interestingly, epimastigotes incubated in media with high heme concentration showed a defective growth and morphology alterations. The toxic effects of heme have been extensively reviewed (15). Heme intercalates in the membranes and promotes lipid peroxidation and membrane-bound proteins oxidation mediated by crosslinking (12), which could explain these alterations. However, despite the observed differences in morphology and growth, epimastigotes incubated with different concentrations of hemin reached a similar intracellular heme concentration. This indicates that *T. cruzi* might exhibit an optimal range of intracellular heme concentration or “heme quota” despite the amount of heme available in the medium. Under heme starvation treatment, intracellular heme dropped



**Figure 5. Overexpression of rTcHTE.His-GFP in *T. cruzi* stimulates amastigotes proliferation.** A, Vero cells were infected with WT parasites or parasites that overexpress rTcHTE.HIS-GFP. 72 h after infection, cells were fixed and stained with Giemsa reagent. Intracellular amastigotes were counted from more than 180 cells for each condition. Statistical significance was determined by Mann-Whitney's test. The experimental data are presented as the mean  $\pm$  S.D. (\*\*\*,  $p < 0.001$ ). B, confocal microscopy images of amastigotes expressing rTcHTE.HIS-GFP. rTcHTE.HIS-GFP (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue).

below the optimal, triggering the expression of TcHTE (allowing its detection by Western blotting). Once the optimal intracellular heme concentration was satisfied, TcHTE protein level decreases (almost undetectable by Western blotting). These results strongly suggest that heme requirement could modulate TcHTE protein level (and mRNA) in the parasite, supporting a direct relation between TcHTE and heme transport activity. We cannot exclude heme degradation as part of a mechanism to control intracellular heme, but genes encoding enzymes with heme oxygenase homology have not been identified in the

genome of *T. cruzi* (16). Presumably, heme degradation via heme oxygenase like activity reported previously (16) would not be enough to get rid of any excess of heme when epimastigotes were exposed at high concentration of hemin. Under this premise, the amount of intracellular heme should be mainly controlled by its transport, with TcHTE playing a role as heme transporter or as an essential part of it.

To clarify if TcHTE responds to signals of extracellular or intracellular heme, we took advantage that *T. cruzi* can selectively import HAs (ZnMP, but not SnMP) (8). Interestingly, although treatment with both HAs reduced intracellular heme levels, only ZnMP decreased TcHTE expression. These results indicate that epimastigotes internalized ZnMP and hemin indistinctly, contributing to the concentration of total metalloporphyrins (heme + ZnMP) and both are sensed by the parasite. On the other hand, SnMP was not detected inside the cell and did not affect TcHTE expression, suggesting that this HA is not sensed or internalized. This is also consistent with the fact that the growth profile was not altered. Therefore, the negative effect observed on epimastigotes growth could be because of the presence of intracellular ZnMP, not to the reduction in heme concentration. These results highlight that epimastigotes only sense intracellular heme independently of the amount of hemin present in the medium and modulate TcHTE expression to adjust heme transport activity. This reinforces the essential role of TcHTE controlling heme homeostasis in *T. cruzi* and that it can tolerate a variation in intracellular heme within the optimal concentration range (2–4 nmol of heme/10<sup>9</sup> cells) without affecting its growth.

The treatment of infected cells with hemin caused a drop in the TcHTE signal in intracellular amastigotes. Assuming that TcHTE mediates heme transport activity in this life stage, the regulation of TcHTE in amastigotes resembles that observed in epimastigotes. Additionally, a significantly higher number of intracellular amastigotes was determined in cell lines infected with the recombinant parasites compared with those infected with WT parasites. Because the presence of recombinant TcHTE increased heme uptake in epimastigotes (8), we hypothesized that rTcHTE.His-GFP also enhances heme uptake in amastigotes, therefore stimulating cellular replication. A similar evidence was found in *L. amazonensis*, in which the deletion of one copy of genomic LHR1 impaired intracellular replication of *L. amazonensis* amastigotes (7). Therefore, it is reasonable to postulate that the ability of intracellular amastigotes to incorporate more heme enhances its replication rate.

Based on the results presented here, we propose a model for the role of TcHTE in *T. cruzi* heme transport that is represented in Fig. 6. In epimastigotes under stationary state of heme flux, TcHTE protein levels remain low. The parasite maintains a constant intracellular heme concentration with low heme import activity. Under heme deprivation (heme starvation), intracellular heme drops and epimastigotes are able to sense it. As a consequence, the expression of TcHTE increases, more protein is assembled in the flagellar pocket region (as heme transporter or part of it), and heme transport activity rises. Once the intracellular heme quota is satisfied, an intracellular signal (still unknown) triggers the mRNA and also the extra-

protein degradation and heme transport activity decreases to stationary level. This model could explain how epimastigotes can response to changes in heme concentration during their path within the insect gut. Epimastigotes experience different heme availability according to the blood feeding sequence of the insect, as well as its location in the insect gut. Triatomines produce hemozoin to reduce the damaging effects of free heme obtained from hemoglobin digestion (17). Despite this, epimastigotes may be in contact with free heme and hemoglobin, which can be imported to supply heme requirements. The constant detection of endogenous TcHTE in intracellular amastigotes is consistent with the fact that available heme in the cytoplasm of host cells is very low (labile heme was estimated in different eukaryotic cells, in yeast 20–40 nM (18), HEK-293 cells ~450 nM (19), IMR90 lung fibroblast cells, ~614 nM (20)) and its presence is necessary to guarantee enough heme uptake in this life cycle stage. The reduction of this protein signal when extra hemin was added to the infected cells supports the proposed model for TcHTE playing a role in heme transport and its regulation along the replicative stages of *T. cruzi*. Based on TcHTE size and its predicted structural features (171 amino acids, 4 predicted transmembrane domains (8)), it should assemble as a homotrimer to form the channel or pore of the heme transporter. Also, TcHTE could be part of a heterocomplex heme transporter. Nevertheless, we cannot exclude TcHTE acting only as a regulatory subunit of the transporter. In this scenario, when heme transport activity is required, TcHTE is synthesized and assembled to build up or activate the transporter.

In summary, TcHTE responds to intracellular heme, confirming it as a member of HRG family protein. Therefore, it might be renamed as TcHRG. *T. cruzi* epimastigotes can manage intracellular heme concentration by controlling its transport in replicative life cycle stages in which the presence of TcHTE/TcHRG is essential to this process. *T. cruzi* can sense intracellular heme, by a still unknown mechanism, and adjust TcHTE expression and accumulation to promote or reduce heme transport activity. Evidences presented here strongly suggest that TcHTE/TcHRG can form the heme transporter or a relevant part of it. In this scenario, the characterization of heme transport and distribution will contribute to a better understanding of the biology and biochemistry of *T. cruzi*. This, in turn, will enable the identification of novel proteins playing essential roles which might act as possible targets for new drug development against Chagas disease.

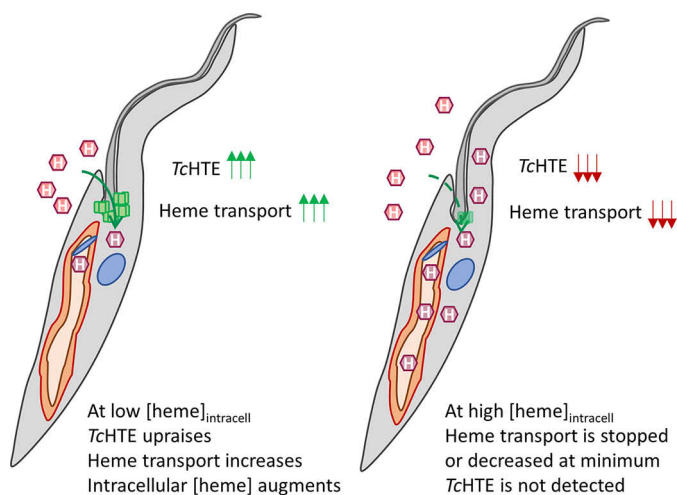
## Experimental Procedures

### Reagents

Dulbecco's modified Eagle medium (DMEM) was obtained from Life Technologies, fetal bovine serum (FBS) from Interne-gocios SA. FBS was heat-inactivated at 56°C for an hour. Hemin, ZnMP (Zn(II) mesoporphyrin IX), and SnMP (Sn(IV) mesoporphyrin IX) were obtained from Frontier Scientific. Hemin, ZnMP, and SnMP stock and working solutions were prepared as described previously (8). Heme concentration in hemin stock solution was confirmed by spectroscopic measurements at 385 nm,  $\epsilon^{385} = 58,400 \text{ M}^{-1} \text{ cm}^{-1}$  (18).



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**Figure 6.** Scheme representing a proposed model for TcHTE/TcHRG function in heme transport. When epimastigotes sense low intracellular heme (left panel), augment TcHTE synthesis and assembly in the flagellar pocket and heme uptake increases. Once intracellular heme raises the optimal quota (right panel), TcHTE is down-regulated and heme import is maintained low, keeping the heme flux at stationary level.

### Parasites and cell lines

All experiments were performed using *T. cruzi* Dm28c strain and all the infections were carried out in Vero cell line (ATCC CCL-81, already available in our laboratory). Epimastigotes were maintained in mid-log phase by periodic dilutions in liver infusion tryptose (LIT) medium supplemented with 10% FBS (LIT-10% FBS) and 5  $\mu\text{M}$  hemin (8), at 28°C. Recombinant epimastigotes were generated as described previously (8). The Vero cell line was routinely maintained in DMEM supplemented with 0.15% (w/v)  $\text{NaHCO}_3$ , and 10% FBS (DMEM-10% FBS) at 37°C in a humid atmosphere containing 5%  $\text{CO}_2$ . During the *T. cruzi* infections, Vero cells were incubated in DMEM supplemented with 2% FBS (DMEM-2% FBS) at 37°C in a humid atmosphere containing 5%  $\text{CO}_2$ . Metacyclic trypomastigotes (WT and recombinant overexpressing rTcHTE.HIS-GFP) were obtained by spontaneous differentiation of epimastigotes at 28°C after 2 weeks of culture without dilution. Metacyclic trypomastigotes were used to infect monolayered Vero cells and to obtain cell-derived trypomastigotes after 5–6 days post infection. Intracellular amastigotes were obtained 2 or 3 days post infection of monolayered Vero cell line with cell-derived trypomastigotes. The homogeneity of the obtained *T. cruzi* forms was evaluated by microscopic observation.

### Antibodies

Rabbit polyclonal anti-TcHTE antibodies were obtained using the p1TcHTE peptide (amino acids 120 to 171) expressed as a TRX.HIS-fusion protein in the vector pET-32a vector (Novagen<sup>®</sup>), following the same strategy we described previously (21). Primers used to amplify p1TcHTE: FP 5'-TCCGGATCCATGATGGCCGCAAAGTGGTG-3' and RP 5'-CCGCTCGAGTTAATGATGATGATGATGATGACCCATAATA-TCTGCGTTCCTTTTCG-3'. The specificity of the antibodies were tested as shown in Fig. S1. All experiments were approved by the Institutional Committee of Animal Care and Use of the

Facultad de Ciencias Bioquímicas y Farmacéuticas (School of Biochemical and Pharmaceutical Sciences), Universidad Nacional de Rosario, Argentina, and conducted according to specifications of the National Institutes of Health guidelines for the care and use of laboratory animals (file number 935/2015).

### Effect of heme on epimastigote growth and TcHTE protein accumulation

Epimastigotes (WT) grown in LIT-10% FBS with 5  $\mu\text{M}$  hemin for 48 h were collected, washed with PBS, and challenged to grow in LIT-10% FBS without (0  $\mu\text{M}$ ) or supplemented with 5, 20, or 50  $\mu\text{M}$  hemin. The number of cells was monitored daily for 14 days. One dilution to the initial condition was made on day 8. On the third and seventh days samples were collected by centrifugation at 2000  $\times g$  for 5 min, washed twice with PBS, and prepared for optic microscopy as described below. Also, aliquots were lysed ( $1 \times 10^6$  parasites/ $\mu\text{l}$ ) with lysis buffer (8 M urea, 30 mM HEPES, pH 8) for Western blot assays.

### Effect of heme on TcHTE protein accumulation in amastigotes

To obtain the intracellular amastigotes, Vero cells were cultured as described above. After 24 h of incubation in DMEM-2% FBS, cells were incubated with trypomastigotes WT in a ratio of 10 trypomastigotes/cell (m.o.i. = 10, multiplicity of infection) for 4 h to let the infection progress. After that, the cells were washed twice with PBS and 2 ml of DMEM-2% FBS was added. 24 h post infection, hemin was added at a final concentration of 20  $\mu\text{M}$ . After 24 h of incubation (with or without extra hemin), cells were washed and intracellular amastigotes and protein extracts were obtained as described previously (21).

### Effect of TcHTE overexpression on amastigote proliferation

To analyze amastigote proliferation, the Vero cell line was plated on 24-well plates with coverslips in DMEM-2% FBS. After 24 h, cells were incubated 4 h with cell-derived trypomastigotes, WT or rTcHTE-overexpressing ones, at m.o.i. = 2. After the infection, the cells were washed twice with PBS and 2 ml of fresh medium were added. 72 h post infection, infected cells were washed with PBS and prepared for Giemsa staining and microscopic analysis as described below. Amastigotes from 183 infected cells for each group were counted by direct observation under microscope.

### Western blotting

Total protein from cell-free extracts obtained from epimastigotes, trypomastigotes, and amastigotes were prepared and processed as described previously (21) with minor modifications. Samples were heated at 42°C for 30 min in loading buffer and  $5 \times 10^6$  cells/well were resolved by electrophoresis on a 15% SDS-polyacrylamide gel. TcHTE detection was performed with rabbit anti-TcHTE antibodies (1:5000). Bound antibodies were detected with peroxidase-labeled anti-rabbit IgG (1:30,000) (Calbiochem), and ECL Prime Western Blotting Detection Kit (GE Healthcare). Loading control was performed with anti-tubulin clone TAT-1 antibodies (a gift from K. Gull, University of Oxford, UK).

### mRNA isolation, RT-PCR, and qRT-PCR

The following samples were used to obtain mRNA from different life cycle stages of *T. cruzi*: Epimastigotes cultured in LIT–10% FBS supplemented with or without 20  $\mu\text{M}$  hemin for 3 days were collected, washed with PBS and cultured in LIT–10% FBS supplemented with 0, 5 or 20  $\mu\text{M}$  hemin for 18 h. Also, trypomastigotes, amastigotes and epimastigotes maintained in LIT–10% FBS supplemented with 5  $\mu\text{M}$  hemin were used for mRNA purification as we described previously, with minor modifications (22). Briefly, the total mRNA was obtained using TRI Reagent<sup>®</sup> (Molecular Research Center, Inc., no. TR 118). The RNA preparations were treated with RNase-free DNase I (Fermentas, Life Sciences), and their quality and concentration were checked following standard procedures (23). Each RNA extraction was carried out by triplicate. cDNAs of *T. cruzi* were synthesized through a RT reaction (M-MuLV, Thermo-Scientific) using 0.5  $\mu\text{g}$  of total RNA. mRNA analysis by quantitative real-time PCR was performed in an Applied Biosystems StepOne<sup>™</sup> Real-Time PCR System Thermal Cycling Block using the SYBR Green fluorescence quantification system (Fermentas). The standard PCR conditions were 95°C (10 min), and then 40 cycles of 94°C (1 min), 60°C (1 min), and 72°C (2 min), followed by the denaturation curve. The primer designs were based on nucleotide sequences of *T. cruzi* CL Brenner Esmeraldo-like genes coding for TcHTE and GAPDH (TriTrypDB accession numbers for TcHTE: TcCLB.511071.190 and GAPDH: TcCLB.506943.50). The sequences of the primers used are listed below. The data were analyzed using StepOne<sup>™</sup> software v 2.1. The -fold change in the expression of the transcripts was obtained using the comparative method ( $\Delta\Delta C_t$ ) (24). The epimastigote stage at 5  $\mu\text{M}$  hemin was used as the reference condition for both genes. Primers for qRT-PCR were TcHTEss 5'-TAATTATTGGGCGGCGGCT-3', TcHTEas 5'-GAAG-TACGAAC TCCCCGTCC-3' (product 235 bp) and GAPDHss 5'-GTGGCAGCACC GGTAACG-3', GAPDHas 5'-CAGG-TCTTTCTTTTGCGAATAGG-3' (product 110 bp). The differences in the transcriptional level among the different stages were compared using one-way ANOVA, followed by Bonferroni's multiple comparison test. For this purpose, the software GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) was used. The significance level (*p* values) was determined with a confidence interval of 95% in a two-tailed distribution.

### Optic microscopy

Epimastigotes were fixed with formaldehyde 3.7% (w/v) in PBS, washed twice with PBS, and settled on poly-L-lysine-coated microscope slides. Parasites were stained with Giemsa reagent for 20–30 min, washed with stabilized water and mounted for microscopic analysis. Infected cells bearing *T. cruzi* amastigotes grown over these coverslips were washed with PBS and fixed with pure methanol for 3 min. Then, the cells were washed again, stained with Giemsa reagent, and mounted with Canada balsam on microscope slides for microscopic analysis

### Confocal microscopy

Samples of epimastigotes and amastigotes were prepared as described previously (8), with minor modifications: infected cells with *T. cruzi* amastigotes grown over coverslips were fixed with pure methanol for 3 min. All the images were acquired with confocal microscopes Nikon Eclipse TE-2000-E2 or Zeiss LSM880 and were processed using the ImageJ software (25).

### Epimastigote growth curve with HAs

Epimastigotes grown in LIT–10% FBS with 5  $\mu\text{M}$  hemin for 48 h were collected, washed with PBS, and challenged to growth in LIT–10% FBS supplemented with 5 and 20  $\mu\text{M}$  hemin, or 5  $\mu\text{M}$  hemin plus 15  $\mu\text{M}$  heme analogs (ZnMP and SnMP). Parasites were maintained in mid-log phase by successive dilution every 2 days and growth was monitored for 9 days. On the third day samples were collected and prepared for confocal analysis as described above or to measure direct fluorescence as described below.

### Heme content analysis

The heme content was quantified by the alkaline pyridine method described in Berry *et al.* (26) with the modification we introduced to perform this assay in epimastigotes (8). Each sample contained  $150 \times 10^6$  epimastigotes. As previously demonstrated, HAs do not interfere with heme quantification (14).

### Fluorescence intensity analysis

$20 \times 10^6$  epimastigotes treated with HAs (obtained from the growth curve) were treated and analyzed as we described previously (8). Fluorescence intensity was measured in a Varian Eclipse fluorometer:  $\lambda_{\text{exc}} = 405$  nm, recording the emission spectra between 450 and 650 nm and analyzing the maximal emission at 578 nm for ZnMP, and 574 nm for SnMP.

### Statistical analysis

The experiments were performed in triplicate, and the data are presented as the mean  $\pm$  S.D. except when informed. All the assays were independently reproduced at least two to four times. Statistically significant differences between groups were assessed using one-way ANOVA followed by Tukey's multiple comparison test (intracellular heme), Mann-Whitney's test (amastigote proliferation) or one-way ANOVA followed by Bonferroni's multiple comparison test (mRNA analysis), as appropriate (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA).

### Data Availability

All data are contained within the article.

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M. L. M., and J. A. C. visualization; L. P., E. T., M. L. M., and J. A. C. methodology; L. P., E. T., M. L. M., and J. A. C. writing-original draft; L. P. and J. A. C. writing-review and editing; J. A. C. conceptualization; J. A. C. supervision; J. A. C. funding acquisition; J. A. C. project administration.

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**Conflict of interest**—The authors declare that they have no conflict of interest with the content of this article.

**Abbreviations**—The abbreviations used are: qRT-PCR, quantitative real-time PCR; SnMP, Sn(IV) mesoporphyrin; ZnMP, Zn(II) mesoporphyrin; LIT, liver infusion tryptose; m.o.i., multiplicity of infection; HA, heme analog; ANOVA, analysis of variance.

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