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Trypanosomatid parasites rescue heme from endocytosed hemoglobin through lysosomal HRG transporters

María Cabello-Donayre, Sophie Malagarie-Cazenave,† Jenny Campos-Salinas,† Francisco J. Gálvez,† Alba Rodríguez-Martínez, Estela Pineda-Molina, Lina M. Orrego, Marta Martínez-García, María P. Sánchez-Cañete, Antonio M. Estévez and José M. Pérez-Victoria*

Instituto de Parasitología y Biomedicina "López-Neyra", CSIC, (IPBLN-CSIC), PTS Granada, Avda. del Conocimiento s/n, Granada 18016, Spain.

Summary

Pathogenic trypanosomatid parasites are auxotrophic for heme and they must scavenge it from their human host. *Trypanosoma brucei* (responsible for sleeping sickness) and *Leishmania* (leishmaniasis) can fulfill heme requirement by receptor-mediated endocytosis of host hemoglobin. However, the mechanism used to transfer hemoglobin-derived heme from the lysosome to the cytosol remains unknown. Here we provide strong evidence that HRG transporters mediate this essential step. In bloodstream *T. brucei*, TbHRG localizes to the endolysosomal compartment where endocytosed hemoglobin is known to be trafficked. TbHRG overexpression increases cytosolic heme levels whereas its downregulation is lethal for the parasites unless they express the *Leishmania* orthologue LmHR1. LmHR1, known to be an essential plasma membrane protein responsible for the uptake of free heme in *Leishmania*, is also present in its acidic compartments which colocalize with endocytosed hemoglobin. Moreover, LmHR1 levels modulated by its overexpression or the abrogation of an *LmHR1* allele correlate with the mitochondrial bioavailability of heme from lysosomal hemoglobin. In addition, using heme auxotrophic yeasts we show that TbHRG and LmHR1 transport hemoglobin-derived heme from the digestive vacuole to the cytosol. Collectively, these results show that trypanosomatid parasites rescue heme from endocytosed hemoglobin through endolysosomal HRG transporters, which could constitute novel drug targets.

Accepted 29 May, 2016. *For correspondence. E-mail josepv@ipb.csic.es; Tel. +34 958181658; Fax +34 958181632. †These authors contributed equally to this work.

Introduction

Protozoa from the trypanosomatid family includes parasites responsible for dramatic neglected diseases in humans and important illnesses in animals. The most important members of this family are *Leishmania* spp., which cause different forms of leishmaniasis in humans and dogs, *Trypanosoma brucei*, responsible for sleeping sickness in humans and nagana in cattle, and *Trypanosoma cruzi*, the etiological agent of Chagas disease in humans. These parasites have digenetic life cycles, alternating between two hosts. In the mammalian host, amastigote forms of *Leishmania* spp. and *T. cruzi* are intracellular, whereas bloodstream forms of *T. brucei* are extracellular. In the insect vector, all forms (*Leishmania* promastigotes, *T. cruzi* epimastigotes and *T. brucei* procyclic forms) are extracellular. Chemotherapy remains the main weapon to fight these diseases, but today's arsenal has many limitations as current drugs are generally toxic and hampered by the increasing problem of drug resistance. Therefore, there is an urgent need to develop new medicines in the fight against these neglected diseases.

A rational approach to discovering new drug targets effective against trypanosomatid parasites consists of exploiting their inability to synthesize the essential compound heme, which they have to salvage from their hosts. This absolute dependence on heme rescue was indeed considered their Achilles' heel more than 80 years ago (Lwoff, 1933). In recent years, some light has been shed on the parasites' heme uptake mechanism. Bloodstream forms of *T. brucei* do not internalize free heme (Vanhollebeke *et al.*, 2008). In contrast, they acquire heme from hemoglobin (Hb), which is taken up through the receptor (TbHpHbR)-mediated endocytosis of haptoglobin (Hp)-Hb complexes (Vanhollebeke *et al.*, 2008). This complex follows the endocytic route to late

endosomes and lysosome, where Hb is digested. The heme moiety released is then exported by an unknown mechanism to the cytosol where it is incorporated into the parasite's hemeproteins, such as sterol 14α-demethylase (Lepesheva *et al*., 2010). Interestingly, although this mechanism facilitates heme rescue from the host, it is also responsible for the human's innate immunity to most African trypanosomes (Vanhollebeke *et al*., 2008; Stodkilde *et al*., 2014). This is because TbHpHbR also recognizes the complex between Hb and Hp-related protein (Hpr), which is present in human HDL particles that also contain the trypanolytic factor apoL1. Only two *T. brucei* subspecies (*T. b. rhodesiense* and *T. b. gambiense*) can evade this defense mechanism and ultimately cause sleeping sickness in humans (Pays *et al*., 2014). In contrast, procyclic forms of *T. brucei*, which reside in the tsetse fly, are able to accumulate free heme but cannot internalize Hp-Hb as they do not express TbHpHbR (Vanhollebeke *et al*., 2008). In the case of *T. cruzi* epimastigotes, heme and Hb internalization take place via different routes (Lara *et al*., 2007). Heme uptake proceeds faster than Hb internalization (Lara *et al*., 2007) and is affected by ABC transporter inhibitors such as cyclosporine A (Lara *et al*., 2007; Cupello *et al*., 2011), whereas Hb is internalized through the endocytic pathway (Lara *et al*., 2007). Finally, promastigotes (Chang and Chang, 1985) and axenic amastigotes (Carvalho *et al*., 2009) of *Leishmania* also take heme from two sources, heme and Hb, which can be also used as a source of iron (Carvalho *et al*., 2009). These compounds are internalized through two independent routes, the faster mechanism is the one involving free heme (Campos-Salinas *et al*., 2011). Hb is captured by receptor-mediated endocytosis (Sengupta *et al*., 1999; Krishnamurthy *et al*., 2005) and trafficked to the lysosome in a process mediated by Rab5 (Singh *et al*., 2003) and Rab7 (Patel *et al*., 2008). Heme is then released from digested Hb (Patel *et al*., 2008), it exits the lysosome by an unknown mechanism and reaches the mitochondria, with the ABC transporter LABCG5 playing an essential role in this trafficking activity (Campos-Salinas *et al*., 2011). This pathway is an attractive therapeutic target: not only *Leishmania* requires degradation of endocytosed Hb for their growth (Patel *et al*., 2008; Campos-Salinas *et al*., 2011; Guha *et al*., 2013), but also the Hb receptor-encoding DNA is a promising vaccine candidate for human use to prevent leishmaniasis (Guha *et al*., 2013). The essential mitochondrial ABC transporter LmABCB3, responsible for cytosolic iron/sulfur clusters biogenesis, also plays a role in mitochondrial heme metabolism (Martinez-Garcia *et al*., 2016). Regarding free hemin, it is imported by the heme transporter LHR1, described in *L. amazonensis* (Huynh *et al*., 2012). The heterologous expression of LHR1 in a *Saccharomyces cerevisiae* strain auxotrophic for heme facilitates yeast growth in the presence of low extracellular hemin concentrations by promoting porphyrin uptake (Huynh *et al*., 2012). LHR1 localizes at the plasma membrane, as expected for a porphyrin importer, but also at the lysosomal compartments of promastigote and amastigote forms of *Leishmania*. This dual localization is also observed in mammalian cells when LHR1 is expressed heterologously (Huynh *et al*., 2012). Whether lysosomal LHR1 is physiologically relevant or is a product of its forced overexpression remains to be seen. Besides, LHR1 is essential for the parasite, and even the ablation of one allele strongly prevents the virulence of the parasite in animal models of cutaneous leishmaniasis, underlining its potential as a new drug target (Huynh *et al*., 2012; Miguel *et al*., 2013; Renberg *et al*., 2015).

LHR1 belongs to the HRG (Heme Response Genes) family of heme transporters, first described in *Caenorhabditis elegans* (Rajagopal *et al*., 2008). In this worm, CeHRG4 is a heme importer at the plasma membrane whereas intracellular CeHRG1 could exports heme from the lysosome to the cytosol (Rajagopal *et al*., 2008; Yuan *et al*., 2012). Similarly, macrophage HRG1 mediates heme export from the phagolysosome into the cytosol during erythrophagocytosis of senescent red cells in mammals (Delaby *et al*., 2012; White *et al*., 2013). All these HRG proteins show low similarity with LHR1 (around 14% identity). *LHR1* syntenic genes are also present in both species of *Trypanosoma* (Huynh *et al*., 2012) and so a role in heme trafficking could be expected.

In this work, we have characterized the TbHRG protein of bloodstream forms of *T. brucei* and analyzed the functional role of lysosomal LHR1 from *Leishmania*. Functional assays in homologous and heterologous systems strongly suggest that both TbHRG and LHR1 are the lysosomal transporters responsible for heme salvage from endocytosed Hb. As these proteins are essential in *T. brucei* and *Leishmania*, and as they present a very low degree of similarity with human HRG1, their specific targeting could represent a novel approach to controlling the neglected diseases derived from these parasites.

Results

Bloodstream forms of T. brucei *express an essential HRG protein in the endolysosomal compartment*

The genome of *T. brucei* presents a syntenic gene of the *Leishmania* heme transporter gene *LHR1*, (Tb927.8.6010) which we have called *TbHRG*. Its protein product TbHRG is 100% identical in the cattle pathogen *T. brucei brucei* and in the human pathogen *T. brucei gambiense*, and only shows a moderate

degree of similarity with other HRG proteins outside the *Trypanosoma* genus (Fig. 1A and *SI Appendix* Table S1): around 22% identity with *Leishmania* HR1, 13% identity with *C. elegans* HRG4, 9% identity with *C. elegans* HRG1 and 16% identity with human HRG1. The related parasite *T. cruzi* also has a LHR1 syntenic gene (with only six amino acid substitutions in the two diploid alleles, TcHRGa and TcHRGb, of the hybrid strain sequenced), and show 32% identity with the *T. brucei* transporter. TbHRG is a 18.1 kDa molecular weight protein and, like other HRG proteins, it is expected to contain four transmembrane domains (Fig. 1A).

We observed, using real-time quantitative PCR (RT-qPCR), that *TbHRG* was constitutively expressed in both stages of *T. brucei*, the expression in the parasite's insect stage being eightfold higher than in bloodstream forms (7.9 ± 1.3 fold, $P < 0.05$). The expression of *TbHRG* in the clinically relevant bloodstream form of the parasite was striking, as *T. brucei* is unable to take up free heme in this stage (Vanhollebeke *et al.*, 2008). Therefore, the physiological function of TbHRG could differ from the one proposed for LHR1, which imports heme from the environment (Huynh *et al.*, 2012). Thus, we analyzed the importance of TbHRG function in bloodstream-form parasites by inducible gene knockdown via RNA interference (RNAi). After adding the inducer doxycycline, the decreased expression of the gene (66%, $P < 0.005$) correlated with an arrest in the parasite's growth (Fig. 1B, left). This growth defect was accompanied by significant phenotypic alterations in the morphology of the induced parasites, as shown in Fig. 1B (right), strongly suggesting that TbHRG is essential in bloodstream-form *T. brucei*, and it is in agreement with high-throughput RIT-seq data (Alsford *et al.*, 2011).

We then analyzed the cellular distribution of TbHRG in *T. brucei*. To that end, we overexpressed a C-terminal GFP-fused version of the protein and analyzed its localization in the parasite using fluorescence microscopy. Figure 1C shows that TbHRG was not observed at the plasma membrane but was actually located intracellularly. The GFP-tagged protein colocalized with Dextran Alexa 647, a fluid-phase endocytosis marker, indicating that it was located in the parasite's endolysosomal compartment. As endocytosed Hb-Hp complexes are trafficked to the parasite late endosome-lysosome (Widener *et al.*, 2007), the essential role of TbHRG could be related to the export of Hb-derived heme to the cytosol.

TbHRG is a heme transporter

To study whether or not TbHRG transports heme, we analyzed the ability of TbHRG to rescue the growth defect of a heme auxotrophic *S. cerevisiae* line. This yeast strain has a deletion in *hem1*, the first gene of the heme biosynthetic pathway (Kohut *et al.*, 2011). As *S. cerevisiae* cannot internalize heme efficiently from the environment, *hem1*Δ yeast must grow in the presence of either ALA, the product of the deleted *hem1* enzyme, or high quantities of hemin, which can enter the cells when at higher concentrations via an unknown mechanism.

We first expressed a GFP-tagged version of TbHRG in a *hem1*Δ yeast strain grown in the presence of ALA. TbHRG-GFP was distributed in both the yeast plasma membrane and in its digestive vacuole, which is equivalent to the trypanosomatid lysosome (Fig. 2A). Control and TbHRG-expressing *hem1*Δ yeasts were then plated in an ALA-free medium with increasing concentrations of hemin. Figure 2B (upper panel) shows that TbHRG was able to rescue the growth of the mutant yeast at low hemin concentrations (0.25 μM), as described for LaHR1 (Miguel *et al.*, 2013) and other HRG proteins (Yuan *et al.*, 2012), strongly suggesting that TbHRG is indeed a heme transporter.

TbHRG transports the heme released after Hb breakdown to the yeast cytoplasm

In yeast, endocytosed Hb is trafficked to the vacuole, where it is digested (Weissman *et al.*, 2008). Subsequently the released heme is not efficiently transported to the cytosol but rather it is degraded in the lumen of the vacuole (Weissman *et al.*, 2008). To assess the possibility that lysosomal/vacuolar TbHRG transports heme produced by the digestion of Hb from these organelles to the cytosol, we evaluated the ability of TbHRG to rescue the growth of *hem1*Δ yeast in the presence of Hb. *Hem1*Δ control yeast cells did not grow in the presence of 0.025 μM Hb (equivalent to 0.1 μM heme), and require higher Hb concentrations before initiating growth (Fig. 2B, lower panel). Contrastingly, mutant yeast cells expressing TbHRG were able to grow at this low Hb concentration and showed a significant improvement in growth at higher Hb concentrations (Fig. 2B, lower panel). Importantly, TbHRG did not increase Hb uptake in the mutant yeast (data not shown). Finally, we also measured TbHRG-mediated bioavailability of Hb-derived heme by measuring the activity of a heme-dependent enzyme, such as catalase. As yeast catalases are hemeproteins regulated by heme and they are not present into the digestive vacuole, catalase activity can be used as an endogenous marker for measuring intracellular heme levels (Kathiresan *et al.*, 2014). We measured yeast catalase activity with the simple assay developed for intact bacterial isolates and human cells (Iwase *et al.*, 2013), consistent in the visualization of the foam produced by the oxygen bubbles generated from the decomposition of hydrogen peroxide by catalase, trapped by a detergent. The foam's height in the test-tubes

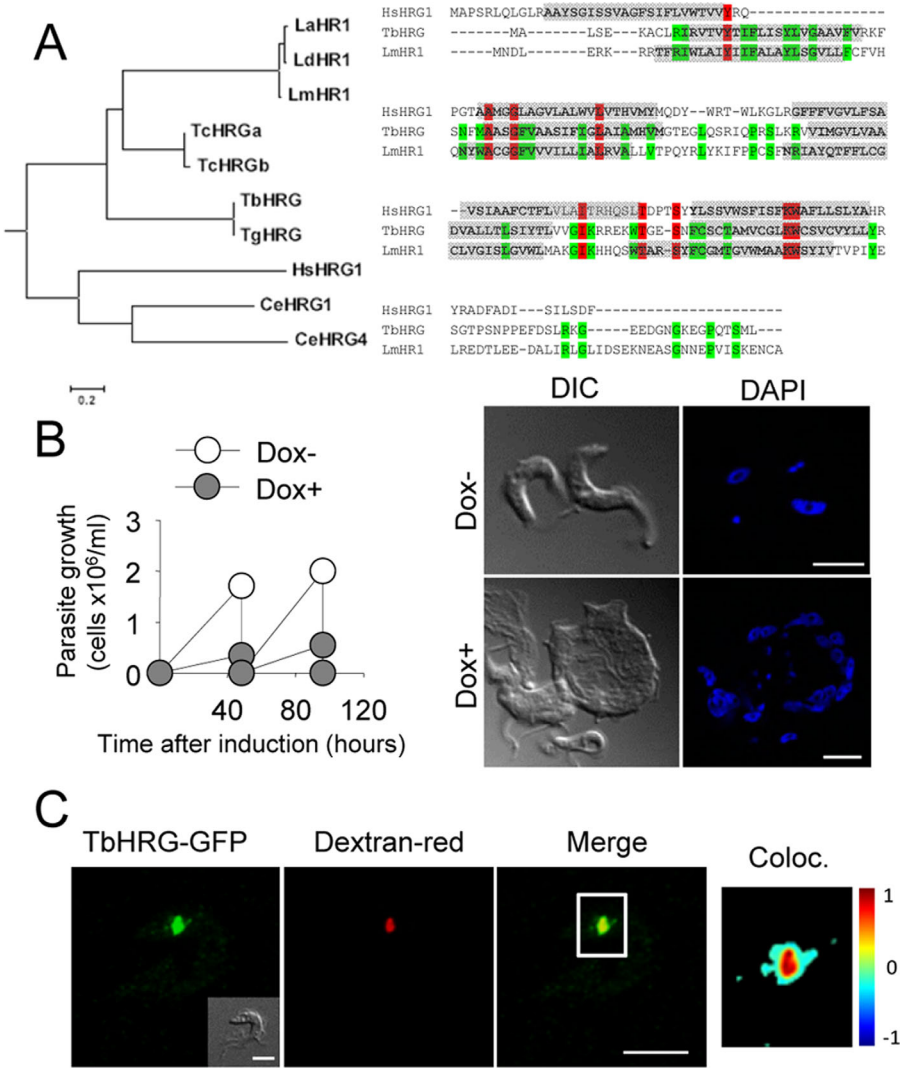

Fig. 1. Bloodstream *T. brucei* parasites express an essential HRG protein located in the endololysosomal compartment of the parasite.
A. The genome of *T. brucei* contains a HRG gene that shows a low similarity with HRG genes from other organisms. *Left*. Phylogenetic analysis of HRG sequences from *L. amazonensis* [LaHR1, (Huynh *et al*., 2012)], *L. donovani* (LdHR1, accession number XP_003861320.1), *L. major* (LmHR1, accession number XP_001683741.1), *T. brucei brucei* (TbHRG, accession number XP_847431.1), *T. brucei gambiense* (TgHRG, accession number XP_011775941.1), *T. cruzi* (TcHRGa, accession number XP_814012.1, and TcHRGb, accession number XP_804283.1), *H. sapiens* (HsHRG1, accession number NP_060312.2) and *C. elegans* (CeHRG1, accession number NP_508690.2 and CeHRG4, accession number NP_502055.1). Aligned protein sequences were subjected to phylogenetic analysis using the Maximum Likelihood method of the MEGA 6 software. *Right*. Amino acids sequence alignment for HsHRG1, TbHRG and LmLHR1 (ClustalW software) highlighting the identical aminoacid shared in these three transporters (red) and in HRG from parasites (green). The transmembrane domains found in all transporters are highlighted in grey.
B. TbHRG is essential in bloodstream *T. brucei*. *Left*. Growth curves of TbHRG-depleted bloodstream trypanosomes after RNAi induction with doxycycline (Dox+) compared with uninduced (Dox−) parasites. Cultures were diluted every 2 days down to 10^4 cells ml^{-1} in order to maintain the cells exponential growth. *Right*. Cell morphology effect in bloodstream trypanosomes upon TbHRG knockdown visualized by DIC optic and fluorescence microscopy of DAPI-stained parasites 48 h after RNAi induction with doxycicline (Dox+). The figure shows parasites representative of a total population with a similar morphology. Scale bar: 5 μm.
C. TbHRG is located in the endolysosomal compartment of the parasite. *T. brucei* cells expressing TbHRG-GFP (green) were incubated at 28°C with the endocytic marker Dextran-Alexa 647 (Dextran-red) for 30 min and then their colocalization (yellow) was analyzed (merge). The figure shows a parasite representative of a total population of parasites with a similar fluorescence pattern. Nomarsky images are shown in the inset. Scale bar: 5 μm. The colocalization color map (right) shows the intensity of colocalization between TbHRG-GFP and the red-labelled dextran marker as indicated by the color bar. In these colour maps, the −1 to +1 heat map depicts the measured Icorr values. Negatively correlated relationships (Icorr values between −1 and 0) are shown in blue-green colors, whereas positive correlations (Icorr values between 0 and 1) are represented by warmer yellow–red colors.

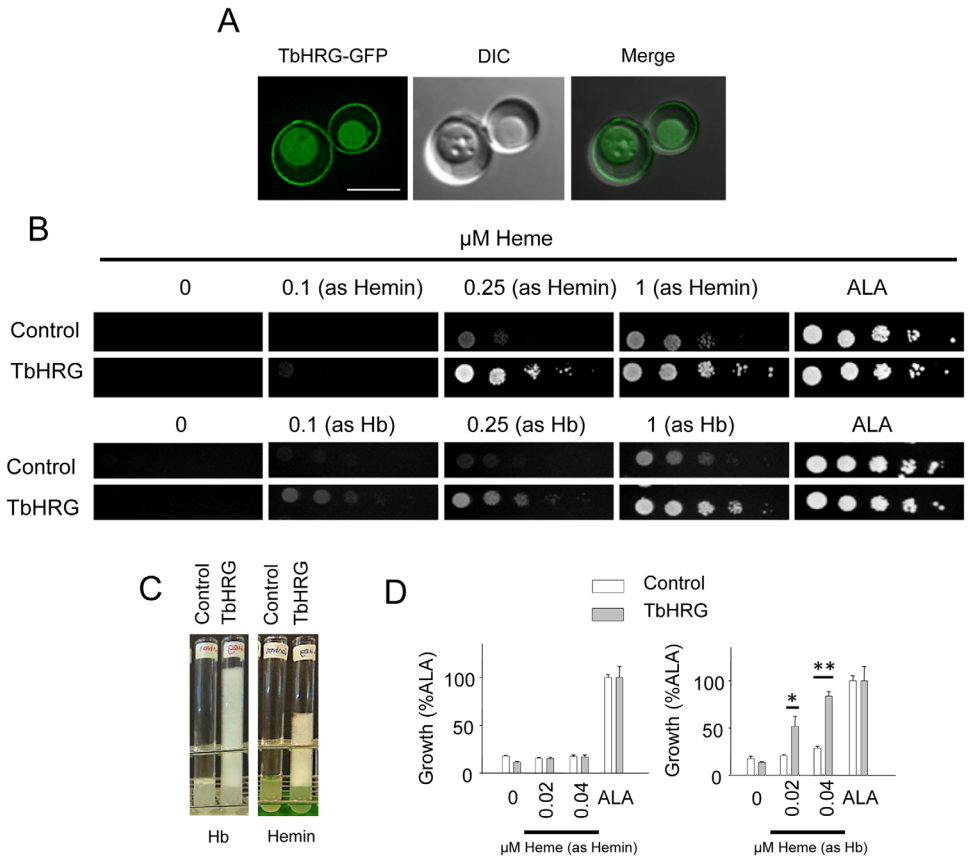

Fig. 2. TbHRG mediates Hb-derived heme transport from the digestive vacuole of yeast to the cytosol.
A. TbHRG heterologously expressed in a heme auxotrophic *S. cerevisiae* strain (*hem1*Δ) shows a double localization at the plasma membrane and the digestive vacuole. The figure shows a yeast cell expressing TbHRG-GFP (green) representative of a total population of cells with a similar fluorescence pattern. Nomarsky images are shown in the central panel. Scale bar: 5 μm.
B. TbHRG enables *hem1*Δ yeast to use endocytosed Hb as a source of heme. *Hem1*Δ yeast transformed with empty plasmid (control) or plasmid containing TbHRG were spotted in 10-fold serial dilutions on plates supplemented with different concentrations of hemin (up) or Hb (down). Positive control: 250 μM ALA.
C. TbHRG increased yeast cytosolic heme levels after Hb endocytosis. Catalase activity, observed as foam formation as described in Materials and Methods, of *hem1*Δ yeast transformed with empty (control) or TbHRG containing plasmid (TbHRG) and incubated with 0.5 μM of Hb (left) or 2 μM hemin (right). Picture representative of three independent experiments.
D. TbHRG-expressing yeast obtained heme more efficiently from Hb than from hemin. *Hem1*Δ yeast transformed with the same plasmids and incubated for 24 h in a liquid medium with the indicated concentrations of hemin (left) or Hb (right). Positive control: 250 μM ALA. The results represent the mean ± SD of three independent experiments with *P* values * < 0.05 and ** < 0.005.

correlates well with the sample's catalase activity (Iwase *et al.*, 2013). Figure 2C shows that *hem1*Δ control yeast incubated in the presence of 0.5 μM Hb had a very low catalase activity, even though the given Hb concentration allowed cell growth of the mutant yeast. By contrast, *hem1*Δ yeast cells expressing TbHRG and incubated under the same conditions produced a significant increase in catalase activity, as shown by the foam developed in the tube (Fig. 2C). These results strongly suggest that vacuolar TbHRG was involved in salvaging heme from internalized Hb. *Hem1*Δ yeast incubation with an equivalent hemin concentration also only resulted in catalase activity when TbHRG was expressed, although at lower levels than those obtained with Hb (Fig. 2C). This supports the idea that plasma membrane TbHRG was also functional.

This catalase assay suggested that Hb-derived heme was used more efficiently by TbHRG-expressing yeast than hemin. To confirm this point, control and TbHRG-expressing *hem1*Δ yeast cells were incubated in a liquid medium containing a low supplement of heme, either as hemin or as Hb, and then their degrees of growth compared. The *hem1*Δ strain, whether expressing TbHRG or not, did not grow at the low hemin concentrations assayed (0.02 and 0.04 μM) (Fig. 2D, left). On the other hand, supplementation with Hb significantly improved the growth of the TbHRG-expressing strain in the presence of only 0.02 μM heme (0.005 μM Hb), and completely restored growth levels at 0.04 μM heme (0.01 μM Hb) (Fig. 2D, right). These results confirm that yeast cells expressing TbHRG were able to obtain heme more

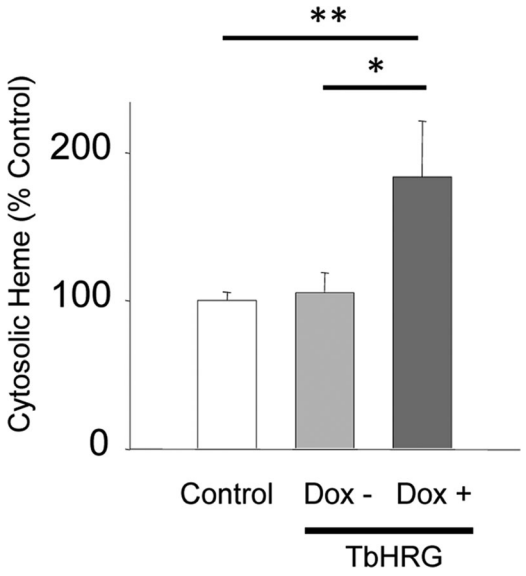

Fig. 3. Lysosomal TbHRG increases cytosolic Hb-derived heme levels in *T. brucei*. Cytosolic heme levels were measured as described in Materials and Methods after incubating the indicated *T. brucei* strains in culture medium including 10% hiFBS as a source of Hp-Hb. TbHRG-GFP expression was induced (Dox+) or not induced (Dox−) with doxycycline. The results represent the mean ± SD of three independent experiments. *P* values * < 0.05 and ** < 0.005.

efficiently from Hb through vacuolar TbHRG than from hemin through plasma membrane TbHRG.

Lysosomal TbHRG increase cytosolic heme levels from endocytosed Hb in T. brucei

Altogether, these results strongly suggested that endolysosomal TbHRG transport Hb-derived heme to the cytosol of bloodstream forms of *T. brucei*. Therefore, we expected that TbHRG overexpressing parasites grew in the presence of Hp-Hb containing serum contain a higher heme concentration in their cytosol. To check it, we measured heme levels in the cytosol after gentle lysis of the parasites overexpressing of not TbHRG, as described in (da Silva Augusto *et al*., 2015). Figure 3 shows that TbHRG overexpressing parasites (after induction with doxicyclin) contained around 85% more cytosolic heme levels than Wt or uninduced parasites ($P < 0.005$).

Leishmania *LmHR1 complements TbHRG deficiency in bloodstream-form* T. brucei

In *Leishmania*, LHR1 is located at the plasma membrane and imports heme from the environment (Huynh *et al*., 2012). Although this function differs from the one proposed here for TbHRG, LHR1 is also located at the parasite's lysosome (Huynh *et al*., 2012) and a similar role in the rescue of heme from endocytosed Hb could be expected. To investigate this point, we first analyzed the ability of *L. major* HR1 (LmHR1) to rescue the essential role of endolysosomal TbHRG in *T. brucei*. Firstly, a C-terminal GFP-tagged version of LmHR1 was cloned in a *T. brucei* expression plasmid and transfected in the TbHRG RNAi *T. brucei* clone described above. Unlike LaHR1 localization in *L. amazonensis* (Huynh *et al*., 2012), LmHR1-GFP was not found at the plasma membrane of bloodstream-form *T. brucei* (Fig. 4A). As with TbHRG-GFP, LmHR1-GFP showed an intracellular location corresponding to the endolysosome compartment, as deduced from its colocalization with fluorescent Dextran (Fig. 4A). Furthermore, the lethal effect produced by RNAi-mediated ablation of *TbHRG* after the addition of doxycycline was mostly circumvented by the expression of LmHR1 (Fig. 4B), indicating that LmHR1 can complement TbHRG function at the endolysosomal compartment of *T. brucei*. RT-qPCR confirmed *TbHRG* underexpression (52%, $P < 0.03$) after the addition of doxycycline.

Vacuolar LmHR1 also rescues heme from endocytosed Hb in yeast

To further confirm that LHR1 could have a similar function to TbHRG, we analyzed its ability to rescue the

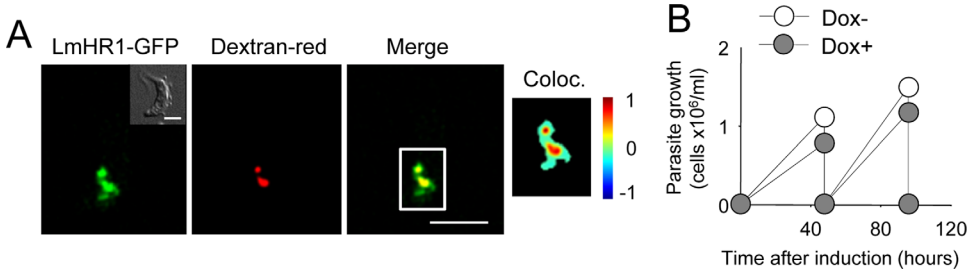

Fig. 4. LmHR1 complements TbHRG function in *T. brucei*.
A. Heterologously expressed LmHR1-GFP is located in the endolysosomal compartment of bloodstream *T. brucei*. Bloodstream *T. brucei* expressing LmHR1-GFP (green) were incubated at 37°C with Dextran-Alexa 647 (Dextran-red) for 30 min and observed using fluorescence microscopy as indicated in Fig. 1C.
B. LmHR1 complements TbHRG function in *T. brucei*. Growth curves of LmHR1-GFP expressing and TbHRG-depleted bloodstream trypanosomes after RNAi induction with doxycycline (Dox+) compared with uninduced (Dox−) parasites, as described in Fig. 1B.

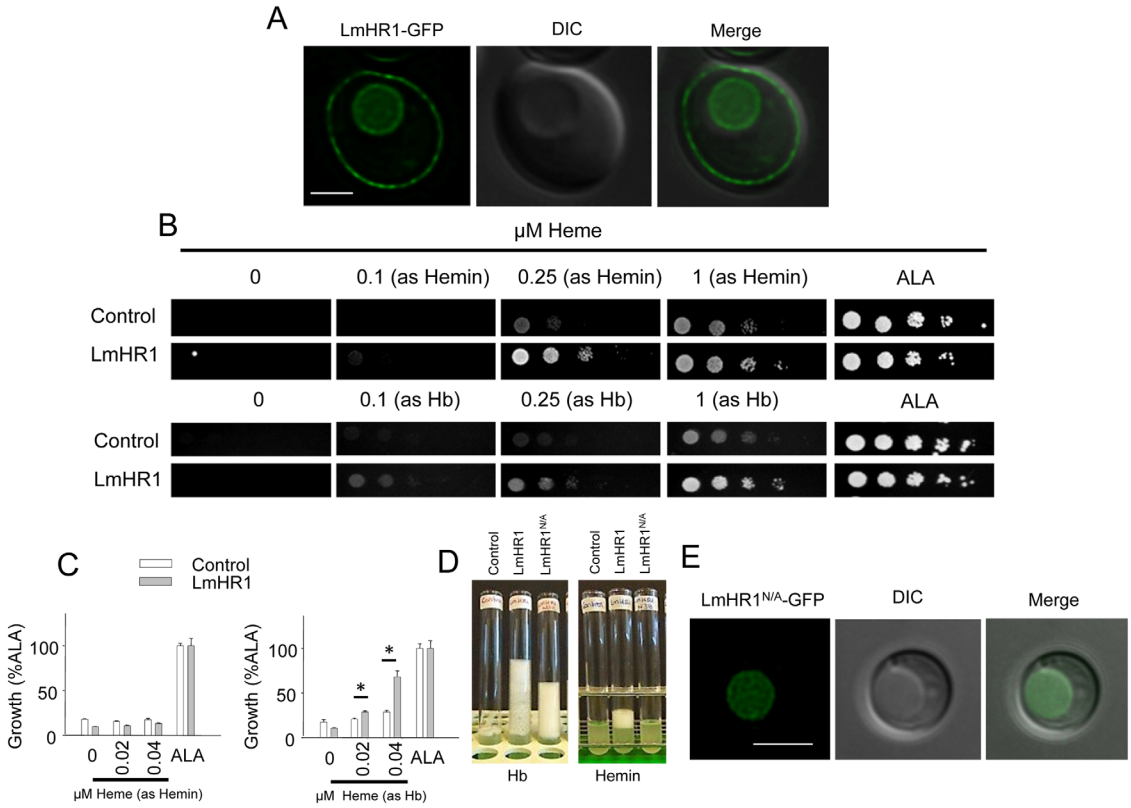

Fig. 5. LmHR1 mediates Hb-derived heme transport from the digestive vacuole of yeast to the cytosol.
A. LmHR1 heterologously expressed in *hem1*Δ yeast shows a double localization at the plasma membrane and the digestive vacuole. LmHR1-GFP is shown in green. Nomarsky images are shown in the central panel. The figure shows a yeast cell representative of a total population of yeast with a similar fluorescence pattern. Scale bar: 5 μm.
B. LmHR1 enables *hem1*Δ yeast to use endocytosed Hb as a source of heme. *Hem1*Δ yeast expressing (LmHR1) or not (control) LmHR1 were spotted in 10-fold serial dilutions on plates supplemented with different concentrations of hemin (up) or Hb (down) as described in Fig. 2B.
C. LmHR1-expressing yeast obtained heme more efficiently from Hb than from hemin. *Hem1*Δ yeast transformed with the same plasmids and incubated for 24 h in a liquid medium with the indicated concentrations of hemin (left) or Hb (right). Positive control: 250 μM ALA. The results represent the mean ± SD of three independent experiments, with *P* value * < 0.05.
(D) LmHR1 mediates Hb-derived heme transport from the yeast digestive vacuole to the cytosol. Catalase activity, measured as described in Fig. 2D, of *hem1*Δ yeast expressing LmHR1 or mutant $LmHR1^{N/A}$ or transformed with empty plasmid (control) and incubated with 0.5 μM of Hb (left) or 2 μM hemin (right). Representative picture of three independent experiments.
(E) Mutant $LmHR1^{N/A}$ is located in the digestive vacuole of *hem1* Δ yeast. $LmHR1^{N/A}$-GFP is shown in green and Nomarsky images are shown in the central panel. The figure shows a yeast cell representative of a total population of yeast with a similar fluorescence pattern. Scale bar: 5 μm.

growth of *hem1*Δ yeast in the presence of Hb. We first analyzed LmHR1-GFP localization in this yeast strain. Fluorescence microscopy showed a double localization at both the plasma membrane and the vacuole (Fig. 5A), as observed for TbHRG-GFP. Then we incubated control and LmHR1-expressing *hem1*Δ yeasts in the presence of increasing concentrations of hemin and Hb. The spot growth assay (Fig. 5B) showed that LmHR1 was able to rescue the growth of mutant yeast in the presence of both sources of porphyrin. Heme derived from Hb was again more efficient than hemin, as 0.01 μM Hb (equivalent to 0.04 μM heme) allowed 70% of the control (ALA) growth in liquid medium, whereas 0.04 μM hemin had no effect in this LmHR1-expressing *hem1*Δ strain (Fig. 5C). As observed for TbHRG, LmHR1 expression did not increase Hb uptake in the mutant yeast (data not shown). Finally, the LmHR1 role in the rescue of Hb-derived heme was measured following the catalase assay described above. Results shown in Fig. 5D indicate that LmHR1 expression significantly increased the catalase activity of yeast incubated in the presence of 0.5 μM Hb. As observed for TbHRG, catalase activity of LmHRG-expressing yeast was higher when the source of heme was Hb rather than free hemin (Fig. 5D).

During the initial cloning of *LmHR1* in yeast expressing plasmids, one clone showed point mutations, probably caused by PCR amplification, that changed amino acid N38, located between TM1 and TM2, to Ala ($LmHR1^{N/A}$). This residue was not included in the extensive mutagenesis analysis performed in LaHR1 (Renberg *et al.*, 2015), and therefore its role was unknown.

Although this mutant was originally discounted, we observed that the protein was well expressed but localized exclusively to the digestive vacuole (Fig. 5E). If this mutation did not affect the functionality of LmHR1, it could be useful in terms of definitively assigning its role at the vacuole. Indeed, when incubated in the presence of 0.5 μM Hb, $LmHR1^{N/A}$-expressing *hem1*Δ yeast cells produced significant catalase activity (Fig. 5D). These results indicate that vacuolar LmHRG can indeed export heme from the digestive yeast vacuole. Contrastingly, hemin supplementation did not increase catalase activity in $LmHR1^{N/A}$-expressing *hem1*Δ yeast (Fig. 5D), in agreement with its absence at the plasma membrane.

Lysosomal LmHR1 rescue heme from endocytosed Hb in L. major

Finally, we analyzed LmHR1's involvement in heme salvage from Hb in *L. major*. A GFP version was initially expressed in *L. major* in order to study the protein's localization. As with LaHR1 (Huynh *et al*., 2012), LmHR1 was present at the plasma membrane, the flagellar pocket and the endolysosomal compartment (colocalization with FM4-64 at 28°C) (Fig. 6A). Intracellular LmHR1 also colocalized with endocytosed Hb, which was added as PPIX-ApoHb, a fluorescent Hb derivative in which heme has been replaced by its immediate precursor PPIX (Fig. 6B). We then studied the role of LmHR1 with respect to porphyrin uptake using a non-tagged version of the transporter. In contrast with the results published with LaHR1 in *L. amazonensis*, in our experiments LmHR1 overexpression did not lead to a significant increase in fluorescent porphyrin uptake in *L. major* (Fig. 6C). To further explore the role of LmHR1 in heme uptake, we deleted one allele of *LmHR1* and replaced it with a hygromycin resistance gene cassette (*SI Appendix* Fig. S1). As described in *L. amazonensis*, we could not produce a double KO line, suggesting that the gene is also essential in *L. major*. This heterozygous knock out line ($LmHR1^{+/-}$) accumulated 60% less fluorescent porphyrin than control cells (Fig. 6C). When an ectopic LmHR1 was expressed in $LmHR1^{+/-}$ cells the parasite's porphyrin uptake capacity was fully restored (Fig. 6C), providing supporting evidence of LmHR1's role in heme uptake. Unlike *T. brucei*, *Leishmania* has a ferrochelatase gene and can therefore insert iron into the precursor PPIX in order to synthesize heme. Thus, the bioavailability of porphyrins from endocytosed Hb could be measured by analyzing the *de novo* heme formation at the mitochondria of parasites incubated with the Hb derivative PPIX-ApoHb (Campos-Salinas *et al*., 2011). Figure 6D shows that heme formation correlated with LmHR1 levels. Thus, LmHR1 overexpression increased heme formation from PPIX-ApoHb by threefold, whereas the deletion of a *LmHR1* allele reduced it by 50%. $LmHR1^{+/-}$ parasites complemented with LmHR1 showed similar levels of heme formation to those of the control cells. As observed in yeast, LmHR1 levels did not affect the endocytosis of Hb in *Leishmania* (data not shown).

Discussion

An Achilles' heel of trypanosomatid parasites is their absolute dependence on scavenging heme from their hosts. Proteins involved in the salvage of this porphyrin could therefore constitute new drug targets for the treatment of the neglected diseases produced by such parasites. In this paper we unravel the mechanism used by *T. brucei* and *L. major* to salvage heme from endocytosed Hb.

Bloodstream forms of *T. brucei*, the clinically relevant form of the parasite, obtain heme exclusively through endocytosis of Hb-Hp complexes (Vanhollebeke *et al*., 2008). *Leishmania*, on the other hand, can obtain heme by its direct import through the essential porphyrin transporter LHR1 (Huynh *et al*., 2012), a protein from the HRG family (Rajagopal *et al*., 2008), or via endocytosis of Hb (Sengupta *et al*., 1999). In both parasites, heme released after the lysosomal breakdown of Hb must cross the lysosome membrane before being incorporated into hemeproteins. Here, we provide evidence which strongly suggests that HRG transporters from *Trypanosoma* and *Leishmania* are responsible for this essential step. Firstly, both LmHR1 and the product of its syntenic gene in *T. brucei*, TbHRG, are localized in endolysosomal compartments of *Leishmania* and bloodstream forms of *T. brucei*, respectively, where internalized Hb is trafficked and digested (Sengupta *et al*., 1999; Vanhollebeke *et al*., 2008). In the case of *Leishmania*, the protein is also expressed in the plasma membrane, where it has been proposed to transport heme from the environment (Huynh *et al*., 2012). A dual localization of LaHR1 in plasma membranes and acidic compartments was also observed when the protein was expressed in HeLa cells (Huynh *et al*., 2012), when TbHRG and LmHR1 were expressed in yeast or in the case of human HRG1 (O'Callaghan *et al*., 2010). Interestingly, LmHR1 has the same exclusive intracellular localization as TbHRG when expressed in bloodstream forms of *T. brucei*. Secondly, both TbHRG and LmHR1 [as LaHR1 (Huynh *et al*., 2012)] mediate heme transport in yeast, as they rescue the growth defect of a heme auxotrophic yeast strain in the presence of low hemin concentrations. Similar rescue phenotypes of the mutant yeast strain have also been described for the HRG proteins CeHRG1, CeHRG4 and HsHRG1 (Yuan *et al*.,

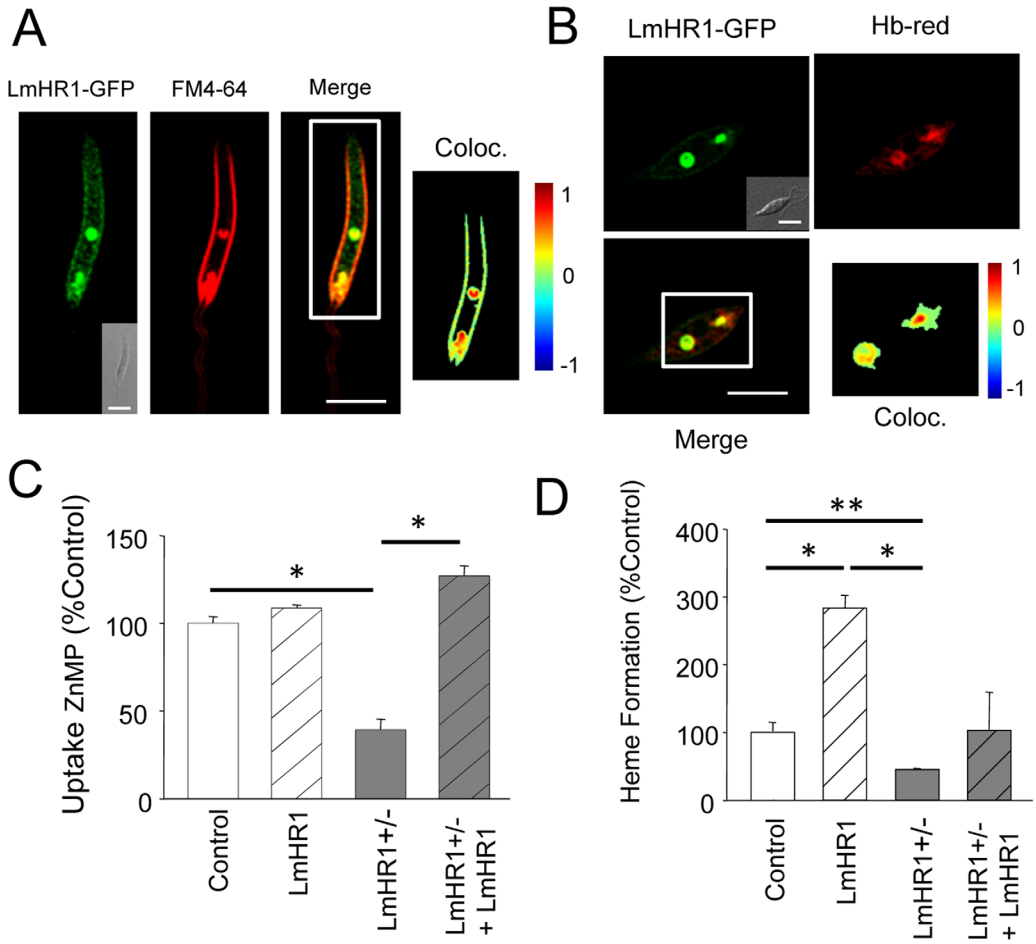

Fig. 6. LmHR1 rescues heme from endocytosed Hb in *L. major* promastigotes.
A. LmHR1 has dual localization at the plasma membrane and the endolysosomal compartment of *Leishmania*. *L. major* promastigotes expressing LmHR1-GFP (green) were incubated at 28°C with 5 μM of FM4-64 (red), a probe to stain the plasma membrane and the endocytic pathway, and observed using fluorescence microscopy as indicated in Fig. 1C.
B. Intracellular LmHR1 colocalized with endocytosed Hb. *L. major* promastigotes expressing LmHR1-GFP (green) were incubated at 28°C in the presence of 10 μM of the fluorescent Hb derivative PPIX-ApoHb (Hb-red) for 30 min and observed using fluorescence microscopy as indicated in Fig. 1C.
C. LmHR1 role in porphyrin uptake in *Leishmania*. The indicated strains of *L. major* promastigotes were incubated with 10 μM ZnMP for 10 min and their intracellular fluorescence analyzed by flow cytometer. Control: parasites with endogenous LmHR1 levels; LmHR1: parasites overexpressing LmHR1; $LmHR1^{+/-}$: parasites with a *LmHR1* allele eliminated; $LmHR1^{+/-}$ + LmHR1: $LmHR1^{+/-}$ parasites transfected with the LmHR1 containing plasmid. The results represent the mean ± SD of three independent experiments. *P* values * < 0.007.
D. LmHR1 mediates Hb-derived heme transport from endolysosome to the cytosol in *L. major*. The mitochondrial synthesis of heme from its precursor PPIX in the form of PPIX-ApoHb was measured as described in Materials and Methods after incubating the indicated *L. major* strains with 0.125 μM PPIX-ApoHb. The results represent the mean ± SD of three independent experiments. *P* values * < 0.005 and ** < 0.0005.

2012). Thirdly, vacuolar TbHRG and LmHR1 are functional as they also rescue the growth of the aforementioned mutant yeast strain when hemin was substituted by Hb, which is known to reach the vacuole after its endocytosis (Weissman *et al.*, 2008). The capacity of these HRG proteins to use Hb as a source of heme in yeast was further confirmed by the fact that they promote catalase activity, which is used as a heme-containing reporter enzyme, following yeast incubation with Hb. This was confirmed using a LmHR1 mutant that localized exclusively to the vacuole and that also facilitated the use of heme derived from Hb. Finally, overexpression of lysosomal TbHRG increases cytosolic heme levels from endocytosed Hp-Hb in *T. brucei* and lysosomal LmHR1 promotes the bioavailability of porphyrins bound to endocytosed Hb in *Leishmania* parasites. Furthermore, TbHRG function in *T. brucei* can be complemented by LmHR1 expression. Therefore, it is likely that both proteins have a similar role in endolysosomal compartments rescuing heme from endocytosed Hb. Similarly, during erythrophagocytosis mammal HRG1 is responsible for heme transport from the phagolysosome of macrophages to the cytosol (Delaby *et al.*, 2012; White *et al.*, 2013), whereas CeHRG1 has been

proposed to facilitate intracellular heme availability through endolysosomal compartments in *C. elegans* (Rajagopal *et al.*, 2008).

The presented data significantly advance our understanding of the mechanisms by which heme auxotrophic trypanosomatid parasites salvage heme from their hosts (Fig. 7). Thus, bloodstream-form *T. brucei* endocytoses Hp-Hb complexes through the Hb-Hp receptor (Vanhollebeke *et al.*, 2008). Hb is then trafficked through the endocytic pathway, digested in the endolysosomal compartment and the free heme released is transported by TbHRG to the cytosol (this work), where it is incorporated in hemeproteins such as sterol 14α-demethylase (Lepesheva *et al.*, 2010). *Leishmania* can internalize heme through plasma membrane LHR1 (Huynh *et al.*, 2012) or it can also use heme bound to Hb (Chang and Chang, 1985; Campos-Salinas *et al.*, 2011). Hb bound to its receptor, a hexokinase, is endocytosed (Sengupta *et al.*, 1999; Krishnamurthy *et al.*, 2005) and routed to the late endosomes/lysosomes where it is degraded and releases the heme moiety (Singh *et al.*, 2003; Patel *et al.*, 2008). Intracellular LHR1, located at the endolysosomal compartment, then export free heme to the cytosol (this work). The ABC transporter LABCG5 probably also plays a role at this point, as it has been shown to be involved in the traffic of Hb-derived heme to the mitochondria (Campos-Salinas *et al.*, 2011). LABCG5 is located in a different vesicular network unrelated to the endocytic route and, therefore, it does not promote heme export from the lysosomes (Campos-Salinas *et al.*, 2011). However, at its location, LABCG5 could take up the heme exported by LHR1 from the endo-lysosomes where Hb is digested (Campos-Salinas *et al.*, 2011), although this possibility still requires investigation. Finally, the mitochondrial ABC transporter LABCB3, which is involved in the maturation of cytosolic iron/sulfur clusters, also promotes mitochondrial heme biogenesis from host precursors (Martinez-Garcia *et al.*, 2016).

The absence of HRG proteins at the plasma membrane of bloodstream *T. brucei* could be due to the low availability of free heme in the human serum, compared to Hp-Hb levels (Thomsen *et al.*, 2013). In contrast, TbHRG (and LHR1) localizes to the plasma membrane and endolysosomes in procyclic *T. brucei* parasites (M. Cabello-Donayre and J.M. Pérez-Victoria, unpublished results), suggesting that this stage of the parasite could also take up free heme released after blood digestion inside the tsetse fly. Likewise, the double localization of HRG proteins at the plasma membrane and intracellular compartments observed in *Leishmania* amastigotes and promastigotes (Huynh *et al.*, 2012) could be also related to the exposition to both, free porphyrins and Hb, in the macrophage phagolysosome and inside the insect vector.

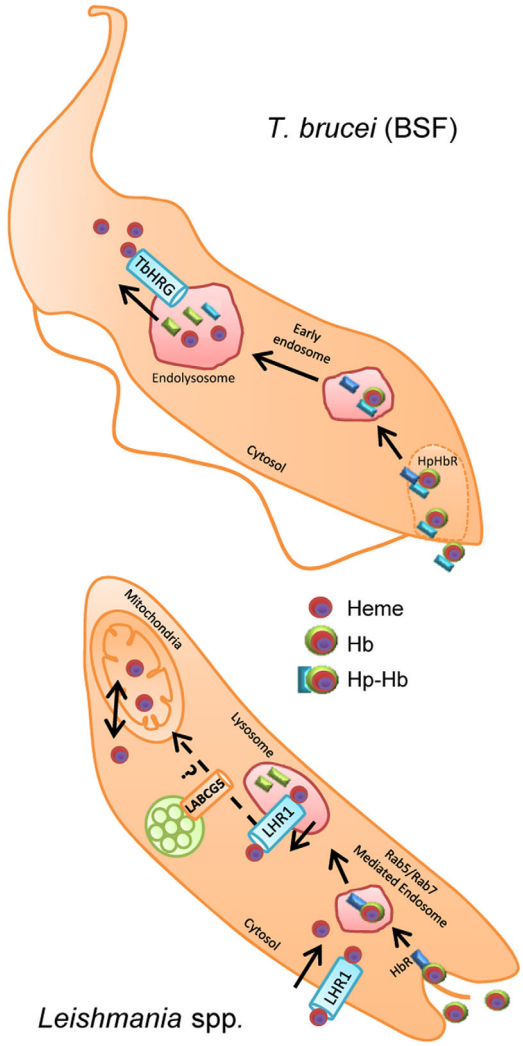

Fig. 7. Proposed models for the role of HRG transporters in bloodstream *T. brucei* and *Leishmania* spp. Bloodstream forms of *T. brucei* endocytose Hp-Hb complexes through the Hp-Hb receptor (HpHbR). Hb is then trafficked through the endocytic route and digested in the endolysosomal compartment releasing the heme moiety. Heme is subsequently exported to the cytosol through TbHRG. In *Leishmania* promastigotes, heme could enter the cells through plasma membrane LHR1. In addition, Hb is internalized by receptor (HbR) mediated endocytosis and trafficked through the endocytic pathway to the lysosome, where Hb is degraded and heme released. This heme is exported from the lysosome by intracellular LHR1 and reaches the mitochondria with the participation of the ABC transporter LABCG5, which is located in a vesicular network unrelated to the endocytic route.

We have also shown that TbHRG is essential for the clinically relevant bloodstream forms of *T. brucei*. The only source of heme in this stage of the parasite's lifecycle is considered to be the receptor (HpHbR)-mediated endocytosis of Hp-Hb complexes (Vanhollebeke *et al.*, 2008), a process well characterized because of its relation with humans' natural immunity against many trypanosome species (Vanhollebeke *et al.*, 2008; Vanhollebeke and Pays, 2010; Pays *et al.*, 2014). The abrogation of TbHRG

expression hampers this means of salvaging heme from the infected host and can explain why it is essential. However, when knocking out the *TbHpHbR* gene, the growth of the trypanosomes has been shown to be severely compromised (Vanhollebeke *et al*., 2008) or slightly affected (DeJesus *et al*., 2013), in either case suggesting that heme scavenging through HpHbR may not be essential for the parasite (DeJesus *et al*., 2013). It has therefore been suggested that *T. brucei* HpHbR$^{-/-}$ parasites employ other heme uptake mechanisms which compensate for the loss of the Hp-Hb receptor (DeJesus *et al*., 2013). This possibility remains to be proven, but the essential nature of TbHRG suggests that this putative alternative mechanism for heme scavenging also relies on the traffic of any form of heme to the parasite's endolysosome, requiring TbHRG for heme export to the cytoplasm. LHR1 is also essential for *L. amazonensis* (Huynh *et al*., 2012) (and probably for *L. major*) and required for the parasite's virulence in animal models (Miguel *et al*., 2013; Renberg *et al*., 2015). Although we have not analyzed the role of the syntenic *HRG* gene of *T. cruzi*, an essential role could also be expected for TcHRG, as *T. cruzi*, responsible for Chagas disease, is also auxotrophic for heme and performs Hb endocytosis (Lara *et al*., 2007). The low similarity between trypanosomatid HRG proteins and the unique human HRG protein (14–18% identity) could enable the development of specific inhibitors against parasite transporters. Therefore, the specific targeting of trypanosomatid HRG proteins represents a promising route for controlling the neglected diseases produced by the aforementioned parasites.

In conclusion, we show that heme auxotrophic trypanosomatid parasites rescue heme from endocytosed Hb through endolysosomal HRG transporters. As these proteins are essential and present a low degree of similarity with their human orthologue, their pharmacological targeting could constitute a novel method to fight these parasites.

Experimental procedures

Chemical compounds

DAPI dihydrochloride, MES hydrate, yeast synthetic drop-out medium supplements without uracil, lithium acetate dihydrate, Triton X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol), Hb and hemin were all obtained from Sigma. PPIX-Na and ZnMPIX were from Frontier Scientific. Dextran Alexa 647 (10,000 MW) and FM4-64 were supplied by Molecular Probes (Invitrogen). Agar was from Intron Biotechnology.

Strains, culture conditions DNA constructs and cell transfection

'Single marker' *T. brucei* Lister 427 bloodstream cell line S16 (Wirtz *et al*., 1999) was cultured HMI-9 medium containing 10% heat inactivated fetal bovine serum (hiFBS) as described in (Carvalho *et al*., 2015). *T. brucei* 449 procyclic cells (Biebinger *et al*., 1997) were grown in SDM-79 medium containing 10% hiFBS and 7.5 μg ml^{-1} hemin. Cells were transfected and selected for the corresponding resistance drugs as described in (Clayton *et al*., 2005). TbHRG from *T. brucei* (Tb927.8.6010) was isolated from genomic DNA of *T. brucei* by PCR using primers pairs P6 and P7 (see all primers used in Table S2), cloned in fusion with GFP (C-terminal) into the expression vector pLEW100 (Wirtz *et al*., 1999). RNAi studies were performed as described in (Clayton *et al*., 2005). Briefly, a fragment of *TbHRG* was PCR-amplified (primer pairs P1 and P2) and cloned into p2T7Blue to yield p2T7-TbHRGi. Bloodstream trypanosomes were transfected with linearized p2T7-TbHRGi and selected in the presence of 5 μg ml^{-1} hygromycin. RNAi was induced with 1 μg ml^{-1} doxycycline. Trypanosomes containing p2T7-TbHRGi were also transfected with the inducible expression plasmid pLEW100 containing LmHR1 (LmjF24.2230) fused to GFP (C-terminal). LmHR1 was isolated from genomic DNA of *L. major* by PCR using primers pairs P11 and P12. Promastigote-form *L. major* (MHOM/IL/80/Friedlin) was cultured in RPMI 1640 modified medium supplemented with 10% hiFBS as described in (Arana *et al*., 1998). LmHR1 was isolated by PCR (primers pairs P13 and P14) and cloned into the *Leishmania* expression vectors pXG (Strain B1288) and pXG-/GFP+ (strain B2863) (Ha *et al*., 1996). Promastigotes were transfected with the different constructs and selected for the corresponding resistance drugs as described previously (Parodi-Talice *et al*., 2003). *S. cerevisiae* BY4742 wild-type and *hem1*Δ (BY4742 *hem1*Δ:BY4742,MAT α;his3Δ1;leu2Δ0;lys2Δ8;ura3Δ0;hem1::LEU2) were grown at 30°C in YPD (yeast extract-peptone-dextrose) or appropriate synthetic complete (SC) medium supplemented with 250 μM δ-aminolevulinic acid hydrochloride (ALA), hemin or Hb. TbHRG and LmHR1 were amplified by PCR using primers pairs P3/P4, P3/P5, P8/P9 and P8/P10 before being cloned into the yeast expression vectors pYESDEST52 and pDR (GFP fusion). Yeast was transformed using the lithium acetate medium (Ito *et al*., 1983) and transformants were selected on 2% w/v glucose SC (-Ura) plates.

LmHR1 gene deletion

Targeted gene replacement of the *L. major LmHR1* gene was performed as we have previously described (Martinez-Garcia *et al*., 2016) for the case of the *L. major LmABCB3* gene. Briefly, a targeting DNA fragment was constructed in which the *hyg* gene (flanked by untranslated regions of the *L. major DHFR-TS* gene (248 bp of the 5′-UTR and 869 bp of the 3′-UTR), conferring resistance to hygromycin B, was flanked by *LmHR1* upstream (primers pairs P15 and P16) and downstream (P17 and P18) regions (Fig. S1). The different fragments were amplified by PCR from genomic DNA using the indicated primers, subcloned into pGEM-T vector (Promega), and then assembled in this vector. Log phase *L. major* promastigotes were transfected with 5 μg of the linearized DNA targeting constructions, generated by ApaI and NotI digestion, through use of the Amaxa Nucleofector System (Lonza). Transfected parasites were selected

with 50 μg ml^{-1} hygromycin B and cloned in semi-solid culture medium (1% agar).

Fluorescence microscopy

Bloodstream-form *T. brucei* expressing TbHRG-GFP or LmHR1-GFP was incubated with 2.5 mg ml^{-1} of Dextran Alexa 647 for 30 min at 37°C as described (Engstler *et al.*, 2005). *L. major* promastigotes expressing LmHR1-GFP were incubated with 10 μM PPIX-ApoHb for 60 min at 28°C in HPMI medium [*T. brucei* cannot internalize this free compound as the receptor TbHpHbR only recognizes the complex Hp-Hb (Vanhollebeke *et al.*, 2008)]. *Hem1*Δ yeast expressing TbHRG-GFP or LmHR1-GFP (Wt or mutated) were used in the exponential growth phase. Cells were washed twice in 500 μl of cold phosphate-buffered saline (PBS), fixed and processed by microscope observation. Images were acquired using Olympus epifluorescence and Leica SP5 confocal microscopes, while the stacks ($n = 40$) were deconvolved using Huygens Professional image processing software from Scientific Volume Imaging (http://www.svi.nl). Statistical colocalization analyses were performed on the 3D- sections with Colocalization Colormap software from FIJI (http://www.fiji.sc/Fiji). The index of correlation (Icorr) represents the fraction of positively correlated pixels in the 3D-images. The results show a representative Z- projection image.

Gene expression analysis

Total RNA from the indicated *Trypanosoma* strains was prepared using the total RNA isolation kit (Roche Biochemicals). cDNA was synthesized from 1 μg of total RNA using qSCRIPT^{TM}cDNA Synthesis kit (Quanta Biosciences Inc.) according to the manufacturer's instructions. The cDNA obtained was amplified with primers pairs P19 and P20 for *TbHRG* and primers pairs P21 and P22 for *LmHR1*. Actin (primers pairs P23 and P24) and tubulin (primers pairs P25 and P26) were used as housekeeping genes. Quantitative PCR was performed with iTaq Universal SYBR Green® Supermix (BIORAD). Relative transcript abundance of TbHRG was calculated using CFX Manager Software, with ΔΔCq method (Livak and Schmittgen, 2001), using as control the TbHRG expression in bloodstream forms.

Yeast growth assays

The spot growth assay was performed as described in (Yuan *et al.*, 2012) but also including the indicated Hb concentration on the plates. Control, TbHRG- and LmHR1-expressing *hem1*Δ yeast were spotted in each plate. The liquid assay was performed similarly, but instead of spot plates heme-depleted cells were suspended in a medium to an OD_{600} of 0.1 and grown with the indicated concentrations of hemin or Hb. OD_{600} was measured after 24 h incubation at 30°C and 180 rpm.

Catalase reporter assay

Catalase activity was measured as described in (Iwase *et al.*, 2013) but with some modifications. Briefly, yeast cells were cultured for 24 h without ALA to deplete heme, and then incubated for 16 h in the absence or presence of either 0.5 μM Hb or 2 μM hemin. Washed yeast cells were then adjusted to a OD_{600} of 5 in 1 ml of H_2O. 300 μl of 1% Triton X-100 and 300 μl of 30% hydrogen peroxide (Sigma) were then added. Catalase-generated oxygen bubbles were trapped by Triton X100 and observed as a foam, the height of which was proportional to the enzyme activity.

Cytosolic heme levels in bloodstream-form T. brucei

Cytosolic heme levels in bloodstream-form *T. brucei* were measured after gently lysis of the parasites as described in (da Silva Augusto *et al.*, 2015). Briefly, parasites (grown in HMI-9 supplemented with 10% hiFBS as a source of Hp-Hb) were washed and lysed by freezing and thawing (three cycles). The insoluble material was removed by centrifugation at 14,000 g for 10 min and cytosolic heme were measured with the Hemin Assay Kit (Sigma) and normalized for the amount of protein as described in (Martinez-Garcia *et al.*, 2016).

Uptake of ZnMP in Leishmania

Porphyrin uptake in *Leishmania* promastigotes was performed as previously described (Campos-Salinas *et al.*, 2011) but with some modifications. Briefly, washed parasites were suspended in HPMI medium (20 mM HEPES pH 7.25, 132 mM NaCl, 3.5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose) and incubated with 10 μM ZnMP at 28°C for 10 min. Parasites were then washed with ice-cold PBS containing 5% BSA and fixed with 2% paraformaldehyde in PBS. Intracellular fluorescence was measured using flow cytometry (excitation at 405 nm and emission at between 575 and 585 nm) employing a FacsAria Cell Sorter III (Becton Dickinson, USA).

Analysis of de novo synthesized heme in Leishmania

ApoHb-PPIX was prepared as described in (Campos-Salinas *et al.*, 2011). Heme-depleted serum was prepared as described in (Carvalho *et al.*, 2009) after filtration of hiFBS over Amicon Ultra 10,000 MWCO columns (Millipore, Carrigtwohill, Co. Cork, Ireland) to select the low molecular-weight fraction of FBS. Heme depletion was verified by measuring the optical absorbance at 405 nm (Huynh *et al.*, 2012). This heme-depleted serum allows normal *Leishmania* growth only when a heme source (hemin or Hb) is provided (data not shown). Synthesis of heme from its precursor PPIX, in the form of PPIX-ApoHb, in *Leishmania* was performed as previously described (Campos-Salinas *et al.*, 2011) but with some modifications. Briefly, parasites were incubated in culture medium supplemented with 10% heme-depleted serum for 16 h with or without 0.125 μM PPIX-ApoHb at 28°C. Parasites were then washed and lysed by six freeze/thaw cycles with liquid nitrogen before intracellular heme levels were measured using the Hemin Assay Kit (Sigma) and normalized for the amount of protein as above described. *De novo* synthesized heme was the difference

between heme levels measured in parasites incubated in the presence and absence of PPIX-ApoHb.

Statistical analysis

Experiments were performed three times in duplicate. All data are presented as the mean and the error represents the S.D. Statistical significance was determined using Student's t-test. Significance was considered to be $P < 0.05$.

Note added in proof

While our manuscript was under revision, Merli and coworkers reported the characterization of TcHTE, the *T. cruzi* ortholog of TbHRG [Merli *et al*., 2016]. The authors showed that TcHTE is located in the flagellar pocket of the parasites and postulate TcHTE as a protein involved in improving the efficiency of the heme uptake or trafficking in *T. cruzi*.

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