Haem-responsive gene transporter enables mobilization of host haem in ticks

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Received: 26 February 2021
Accepted: 4 August 2021

Subject Area:
cellular biology/microbiology/molecular biology

Keywords:
ticks, HRG, transporter, haem, auxotrophy

Tick-borne disease vectors, notorious blood-feeders and disease-vectors, have lost a part of their genetic complement encoding haem biosynthetic enzymes and are, therefore, dependent on the acquisition and distribution of host haem. The parasites therefore need to salvage host haem and distribute it across their cells and body tissues in order to recycle the acquired host haem for their own metabolic demands. Protein components of the molecular network facilitating such haem distribution are, however, poorly understood. Ticks, also, cannot synthesize haem de novo and need to operate a regulated acquisition–distribution–disposal network to allow haem-based metabolism and, at the same time, prevent cytotoxicity resulting from haem overload [2]. Upon blood-feeding, engorged tick females acquire and deposit haem into ovaries to allow embryogenesis in laid eggs. The absence of dietary haem leads to the failure of engorged tick females to reproduce [3]. Such maternal haem is acquired as a liberated by-product of lysosomal digestion of host haemoglobin in tick midgut cells [3,4]. A vast excess of haem gets detoxified through intracellular bioaccumulation of haem within so-called residual bodies/haematin granules/haemosomes [5–7], but a certain amount of haem is translocated into the cytosol, where it primarily gets complexed with haem-binding enzymes, chaperons and transporters. The manner in which haem is translocated into the cytosol of tick midgut digest cells is, however, unknown.

1. Introduction

Several parasites have, through evolution, lost genes encoding haem biosynthetic enzymes, yet they retained and perform haem-based metabolism [1]. The parasites therefore need to salvage host haem and distribute it across their cells and body tissues in order to recycle the acquired host haem for their own metabolic demands. Protein components of the molecular network facilitating such haem distribution are, however, poorly understood. Ticks, also, cannot synthesize haem de novo and need to operate a regulated acquisition–distribution–disposal network to allow haem-based metabolism and, at the same time, prevent cytotoxicity resulting from haem overload [2]. Upon blood-feeding, engorged tick females acquire and deposit haem into ovaries to allow embryogenesis in laid eggs. The absence of dietary haem leads to the failure of engorged tick females to reproduce [3]. Such maternal haem is acquired as a liberated by-product of lysosomal digestion of host haemoglobin in tick midgut cells [3,4]. A vast excess of haem gets detoxified through intracellular bioaccumulation of haem within so-called residual bodies/haematin granules/haemosomes [5–7], but a certain amount of haem is translocated into the cytosol, where it primarily gets complexed with haem-binding enzymes, chaperons and transporters. The manner in which haem is translocated into the cytosol of tick midgut digest cells is, however, unknown.
Haem is amphipathic in nature and, in the cellular environment, partitions into membranes and the protein pool. Predictions for haem transport range from postulating exclusive proteinaceous transport systems to autonomous diffusion across lipid membranes \[8,9\]. The former method of transport is mediated by haem transporters, many of which are recent theories in various eukaryotic organisms \[10\]. Solute carrier protein SLC48A1, known as haem-responsive gene 1 protein (HRG1), has been implicated in such haem transport in many organisms ranging from unicellular kinetoplastid parasites \[11–13\], parasitic nematodes, trematodes, and molluscs, showed strong support for their clustering. Phylogenetic analyses nested the parasitic nematodes (Trichinella sp. and Toxocara canis) as the most basal clade in the tree. Species of echinoderms, molluscs, crustaceans and trematodes \(\text{Scoliodon japonicum}\) were positioned as a sister taxa to each other. Additionally, these taxa fell on the root of the split for arthropods and vertebrates. The arthropod branch was further divided into acarids and insects, where our protein sequence clustered with HRG data obtained from other arthropods \[12,13,24\]. The individual groups of invertebrates, such as arthropods (insects and acarids), parasitic nematodes, trematodes and molluscs, showed strong support for their clustering. Phylogenetic analyses nested the parasitic nematodes (Trichinella sp. and Toxocara canis) as the most basal clade in the tree. Species of echinoderms, molluscs, crustaceans and trematodes \(\text{Scoliodon japonicum}\) were positioned as a sister taxa to each other. Additionally, these taxa fell on the root of the split for arthropods and vertebrates. The arthropod branch was further divided into acarids and insects, where our protein sequence clustered with HRG data obtained from other arthropods. Furthermore, all HRG1 data of other tick species, particularly \textit{Ixodes scapularis} and \textit{Amblyomma variegatum}, were grouped together. The tick-branch had robust support and was separated from the whole acarid group.

Interestingly, the HRG1 homologue has not yet been identified in blood-feeding insect species, but was found to be preserved in several non-blood-feeding insect groups (beetles, termites and ants) (electronic supplementary material, figure S1). HRG1 membrane protein was discovered for all the major groups of vertebrates and seemed to be conserved within each group. Nei’s distances, computed by Geneious software, showed the highest level of similarity with the \textit{Ixodes} data (65.3–97.7%), but, interestingly, a high level of difference within the arthropods (acarids 25.9–32.6%, insect 18.2–30.1%) (electronic supplementary material, table S1). Caenorhabditis elegans HRG1 and its \textit{I. ricinus} homologue contained four transmembrane helices, a glutamic acid \(\text{D/E}X_{3}XXLL\) motif, a specific sorting signal directing transmembrane proteins to compartments of the endosomal–lysosomal system \[16,21–23\]. A predicted transmembrane topology of HRG and haem is shown on the cartoon to the right.

### Figure 1
Phylogenetic analysis (a) based on HRG1 protein amino acid sequences of vertebrates and invertebrates with a final length of alignment of 143 aa. The tree was computed by the W-IQ-TREE software. Sequences of protists were used as an outgroup to root the final tree. Statistical support for each branch was obtained from the ML bootstrap. (b) Amino acid sequence alignment of tick (\textit{I. ricinus}), human and \textit{C. elegans} homologues of \textit{iHRG1}. Blue arrows indicate histidine residues implicated in association with haem and red arrows indicate basic amino acid residues of another haem-binding motif \[20\]. Orange arrows indicate dileucine (D/E)XXXLL motif, a specific sorting signal directing transmembrane proteins to compartments of the endosomal–lysosomal system \[16,21–23\]. A predicted transmembrane topology of HRG and haem is shown on the cartoon to the right.

2. Results

2.1. Phylogenetic analysis underscores the unique conservation of tick HRG1 within arthropods

The tick HRG1 homologue, \textit{IrHRG}, was identified (contig ID: Ir4974; protein ID: JAP70263.1) in our RNAseq data from \textit{I. ricinus} midgut transcriptome analysis \[19\]. To assess its relationship with HRG1 amino acid sequence data from other organisms, we performed maximum-likelihood (ML)-based phylogenetic analyses of our dataset encompassing blood-feeding nematodes, insects and vertebrates, with protists being used as an outgroup (figure 1a). We carried out phylogenetic analyses in three independent runs, where all three runs provided identical results. The final tree was rooted using protist sequences that encode HRG1 transporter \[12,13,24\]. The individual groups of invertebrates, such as arthropods (insects and acarids), parasitic nematodes, trematodes and molluscs, showed strong support for their clustering. Phylogenetic analyses nested the parasitic nematodes (Trichinella sp. and Toxocara canis) as the most basal clade in the tree. Species of echinoderms, molluscs, crustaceans and trematodes (Schistosoma japonicum) were positioned as a sister taxa to each other. Additionally, these taxa fell on the root of the split for arthropods and vertebrates. The arthropod branch was further divided into acarids and insects, where our protein sequence clustered with HRG data obtained from other acarids. Furthermore, all HRG1 data of other tick species, particularly \textit{Ixodes scapularis} and \textit{Amblyomma variegatum}, were grouped together. The tick-branch had robust support and was separated from the whole acarid group.

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acid—dileucine endo-lysosomal targeting motif, and key histidine residues that protrude through the second transmembrane domain into the endosomal lumen, where they mediate HRG1 activity [20] (figure 1b). The ‘FARKY’ key motif of \textit{C. elegans} HRG1 of the endosomal lumen-facing C-terminal is, however, highly mutated in \textit{I. ricinus} HRG1 homologue with an ‘SAHHC’ motif. The duplet of basic amino acid residues within the motif, however, is conserved across all homologues examined: ‘FH’ for \textit{I. ricinus}, ‘HR’ for human and ‘RK’ for \textit{C. elegans} HRG1 (figure 1b). As ticks do not encode other likely homologues of \textit{C. elegans} \textit{hr} genes, we further denote \textit{I. ricinus} HRG1 homologue as \textit{IrHRG}.

### 2.2. Expression of \textit{IrHRG} is independent of dietary haemoglobin

Intracellular haem homeostasis in metazoan organisms is facilitated by the haem importer HRG-1 via its transcriptional upregulation at low availability of exogenous haem; hence the name haem-responsive gene (HRG) [16,25]. Our previous transcriptomic study did not indicate any differences in levels of \textit{IrHRG} in midguts of serum- and blood-fed ticks [19]. We re-examined this apparent deviation by RT-qPCR of midguts dissected from ticks fed serum, serum + haemmin, serum + haemoglobin or blood, but did not observe any statistically significant differences in levels of \textit{IrHRG} transcripts (electronic supplementary material, figure S4). To gain a more comprehensive picture of \textit{IrHRG} haem-mediated regulation, we performed a RT-qPCR differential expression analysis of \textit{IrHRG} in all developmental stages of \textit{I. ricinus} ticks, which were artificially fed either serum or blood (figure 2a). In line with our previous transcriptomic data, we did not detect any differential expression of \textit{IrHRG} in whole-body homogenates of blood- and serum-fed larvae and nymphs (figure 2b), nor did we detect differential levels of \textit{IrHRG} in guts (figure 2c) or ovaries (figure 2d) dissected from blood- or serum-fed females. These data suggest that the expression of \textit{IrHRG} transcripts is independent of the presence of haemoglobin haem in the diet.

### 2.3. \textit{IrHRG} rescues growth of Δ\textit{hem}1 \textit{S. cerevisiae} by the enhanced import of exogenous haem

The absence of haem-mediated regulation of \textit{IrHRG} levels raised the question whether \textit{IrHRG}, a distant \textit{CeHRG1} and mammalian homologue [16,18], retained its function as a haem transporter. To examine and verify the presumed function of \textit{IrHRG} as a membrane-localized haem transporter, we used complementation assays with \textit{Saccharomyces cerevisiae} yeasts that do not code for HRG1-related protein homologues [11]. Specifically, we used a genetic mutant yeast \textit{Δhem}1 \textit{S. cerevisiae} unable to produce δ-aminolevulinic acid (ALA) [12], the first committed precursor of haem biosynthesis. This yeast strain is, therefore, a genetic auxotroph unable to produce endogenous haem and needs ALA for growth (figure 3a). The \textit{Δhem1} cells do not grow solely on a sugar source but require
exogenous ALA to be internalized and to drive endogenous haem biosynthesis, resulting in uniform cellular densities irrespective of the presence or absence of HRG1 (figure 3b). Unlike the wild-type strain, which is not efficient in haem uptake, the Δhem1 strain exhibited the increased capacity to internalize haem from the culture medium [26]. However,
when grown under low haemin supplementation (less than 10 μM) [11,20], this strain struggled to grow unless a haem transporter was ectopically expressed. This serves therefore as a powerful assessment of ectopically expressed haem transporters in order to confirm their predicted function. Such a mutant complementation approach has been employed in numerous previous studies [12,18,20,27]. Using strain Δhem1, we also monitored the subcellular localization of IrHRG by expressing it as a fusion protein of IrHRG and C-terminal GFP (IrHRG-GFP). While GFP (empty vector) clearly localized into the yeast cytosol, IrHRG-GFP was predominantly localized to membranes of yeast digestive vacuoles (figure 3c), a membrane compartment equivalent to the lysosome of higher eukaryotes; this is consistent with the presence of the dileucine sorting motif in IrHRG (figure 1b). IrHRG was also partially localized to the plasma membrane (figure 3c). The expression of IrHRG-GFP, monitored by flow cytometry, was identical to that of Leishmania major HRG-GFP construct LmHRG_GFP and GFP itself (electronic supplementary material, figure S2). This assumes IrHRG to be expressed to a similar extent as LmHRG_GFP and GFP in the yeast cell, thus permitting a direct comparison of haem transport capacity. To assess the transporter function of IrHRG, a stop-codon containing IrHRG insert was cloned into the plasmid to ectopically produce a non-tagged (no-GFP) IrHRG.

To assess the haem transporting capacity of IrHRG, we evaluated the growth rescue ofΔhem1 cells with low haemin supplementation (0.1–0.5 μM), either on solid agar plates (figure 3d) or in liquid culture (figure 3e). Indeed, the ectopic expression of IrHRG facilitated enhanced growth of the yeast in a dose-dependent manner corresponding to the haem concentration in the medium, similar to HRG from L. major (LmHRG), which was used as a positive control. To assess the bioavailability of haem, we evaluated the activity of catalase, an intracellular haem-dependent enzyme producing oxygen from the decomposition of hydrogen peroxide. When the cellular suspension was supplemented with detergent, foamy formation was observed upon the addition of hydrogen peroxide. While empty-vector yeasts produced minute amounts of oxygen foam, IrHRG and control LmHRG producing yeast cells produced substantially higher levels of foam when supplemented with haem or haemoglobin (electronic supplementary material, figure S3). In a dose-dependent assay of twofold dilutions, we noted that IrHRG at least doubled the bioavailability of haem (figure 3f).

In a reverse experiment, the Δhem1 yeast growth medium was supplemented with gallium protoporphyrin IX (GaPPIX), a toxic haemin-related analogue, with coordinated Ga3+ ions instead of Fe3+ ions in the tetrapyrrrole centre [28]. The Δhem1 yeasts expressing IrHRG/control LmHRG grown in medium supplemented with 50 μM GaPPIX had lower cellular densities than yeasts with no HRG, as evidenced by growth assays on solid (figure 3g) and liquid (figure 3h) drop-out media. IrHRG thus clearly increased the cellular sensitivity to GaPPIX-mediated toxicity, which was also evident from decreased catalase activity when cells were co-cultured with ALA and GaPPIX (figure 3i). These yeast mutant-derived data clearly illustrate the capacity of IrHRG protein to translocate tetrapyrrrole molecules and to make them available based on cellular demand.

2.4. Blood-feeding confers resistance of ticks to GaPPIX toxicity

To demonstrate the sensitivity of I. ricinus ticks to GaPPIX, analogously to IrHRG-expressing yeasts, we designed a differential feeding assay using the artificial feeding system (figure 4a). While blood-fed ticks were shown to be insensitive to GaPPIX (figure 4b), serum-fed ticks clearly displayed dose-dependent sensitivity to GaPPIX, preventing tick engorgement. Ticks supplied with greater than or equal to 10 μM GaPPIX in dietary serum did not progress through engorgement and achieved negligible weights after 10 days of feeding (figure 4b). This observation indicates that blood-feeding prevents GaPPIX-mediated toxicity as GaPPIX is unlikely to displace acquired and complexed haem in endogenous haem-dependent enzymes. GaPPIX complexing with cellular haem-binding enzymes is enabled, however, when no haem is acquired from the tick diet.
2.5. RNAi-silencing of IrHRG rescues ticks from GaPPIX-mediated toxicity

To verify whether IrHRG plays a role as a porphyrin transporter in vivo, we examined whether RNAi-mediated silencing of IrHRG in ticks (IrHRG-KD) can rescue GaPPIX-mediated toxicity in serum-fed ticks. We managed to silence IrHRG transcripts in the midgut of serum-fed I. ricinus female ticks down to 16% expression levels of controls (figure 5a) and subjected these females to membrane feeding of dietary serum supplemented with 25 µM GaPPIX. We compared the capacity to engorge in IrHRG double-stranded RNA (dsRNA) and gfp dsRNA (non-specific dsRNA control) injected ticks. Indeed, IrHRG silencing rescued ticks from GaPPIX toxicity with IrHRG-KD ticks gaining more weight than GFP controls (figure 5b,c). To corroborate these data, we followed the uptake of fluorescent metalloporphyrin, zinc protoporphyrin IX (ZnPPIX), in midgut digest cells of the IrHRG-KD ticks. While ZnPPIX was evenly distributed across the tick midgut in control ticks, ZnPPIX accumulated in tick midgut digest cells upon RNAi KD (electronic supplementary material, figure S5). The results of these experiments provide strong evidence that IrHRG is the bona fide tetrapyrole transporter.

2.6. RNAi-silencing of IrHRG reduces bioavailability of haem in tick midgut digest cells

During blood-feeding, ticks internalize haemoglobin in their midgut cells, where it is hydrolysed under the acidic conditions of the endo-lysosomal vesicles by a network of mainly cysteine proteases [4]. Endocytosed host haemoglobin becomes associated with large endocytic vesicles ranging from 3–12 µm in Rhipicephalus microplus [29] and less than 1 µm in I. ricinus ticks [30]. Haem is then released from digested haemoglobin and accumulates in lysosomal residual bodies, also referred to as haemosomes [7,29]. From previous studies in other organisms, it is clear that HRG1 proteins localize to membranes of vesicles of the endo-lysosomal system [16,31], which is in line with predicted (figure 1b) and documented (figure 3c) targeting of IrHRG. To assess if IrHRG also participates in haem trafficking within the endo-lysosomal network of the tick digest cells, we followed haem accumulation in semi-thin (figure 6a,b) and ultra-thin (figure 6c,d) sections of tick midgut cells under light microscopy and transmission electron microscopy (TEM), respectively. Tick midgut digest cells of IrHRG-KD ticks formed significantly larger haem-dense vesicles (haemosomes) than those of control ticks (figure 6e). In both cases, haemosomes were found to be associated with digestive vesicles, as identified using anti-cathepsin B-specific antibodies [32] (figure 6f). We have previously characterized a haem-binding glutathione S-transferase (IrGST1) that is specifically expressed in the midgut of blood-fed and not serum-fed I. ricinus ticks [19,33]. Transcription of the ir-gst1 gene is induced by the availability of dietary haem in the cytosol of digestive cells [33]. To examine whether IrHRG facilitates ir-gst1 expression, we performed RNAi-silencing of IrHRG and found that ir-gst1 mRNA levels were significantly reduced in IrHRG-KD ticks compared to
the GFP controls (figure 6g). This result confirmed that haem bioavailability within tick midgut digest cells was mediated by *lrHRG* haem transport.

On the organism level, however, *lrHRG*-KD ticks displayed the comparable capacity to fully engorge and reproduce when fed *in vivo* on full blood [34] (electronic supplementary

Figure 6. Microscopic examination of haem accumulation in digest cells *lrHRG*-KD ticks. GFP control (a,c) and *lrHRG*-KD (b,d) ticks were partially engorged for 5 days. Ticks were dissected and their midguts embedded, sectioned and visualized by toluidine blue staining under light microscopy (a,b) or TEM (c,d); DC, digest cell; Lu, midgut lumen; Hb-C, haemoglobin crystals; DV, digestive vesicle; H, haemosomes; yellow arrows point towards contact sites of haemosomes and digestive vesicles. Scale bar (a,b) indicates 20 µm, scale bar (c,d) indicates 1 µm. (e) Bar graph shows diameters of haemosomes, which were measured by ImageJ from TEM images. Ten haemosomes were measured in five different images of a gut. Each group was represented by three midguts coming from individual ticks. * Indicates p < 0.05 of the t-test analysed by GraphPad Prism. (f) Immuno-gold detection of *I. ricinus* cathepsin B within a midgut digest cell of partially fed adult *I. ricinus* female; DV, digestive vesicle; H, haemosomes; yellow arrows point towards contact sites of haemosomes and digestive vesicles. Scale bar indicates 500 nm. (g) RT-qPCR analysis of *ir-gst1* in adult female midguts dissected at 3 days of feeding and AD (FE—fully engorged ticks); each dataset is derived from RNA extracts of three individual females.
material, figure S6A–C), as well as when fed in vitro low-haemoglobin-containing serum or serum alone (electronic supplementary material, figure S6D–F), indicating high redundancy of a haem transport system in tick tissues.

3. Discussion

Many species of arthropod lineages do not appear to code for HRG homologues. Using BlastP homology searches, we detected HRG homologues in ants, shrimps and ticks, but unexpectedly, no HRG homologue was found in blood-feeding insects. Admittedly, sequence similarity between identified homologues within arthropods or nematodes was very low. Therefore, we cannot rule out the presence of a functional homologue with very distant primary sequence beyond the identification constraints of the BlastP search. In this work, we took a synergistic approach of genetics, biochemistry, and microbiology to elucidate the function of IrHRG in the hard tick *I. ricinus*, the European vector for Lyme borreliosis and tick-borne encephalitis.

Apart from its canonical functioning as an enzymatic cofactor, haem also serves as a signalling molecule regulating the expression of gene transcripts [35]. We have previously described positive regulatory mechanisms in the tick midgut, which were mediated by iron or haem signalling [3,19,33,36]. By contrast, SLC48A1 protein was first discovered through microarray screens in *C. elegans* as a gene with repressed transcription in response to high haem availability in the medium and was denoted as a HRG [16,25]. Here, we showed that *IrHRG* transcript levels were not elevated during tick feeding of a haemoglobin-depleted diet (i.e. serum feeding). As the haem content in the *C. elegans* diet is not predictable, regulatory mechanisms need to be employed to secure haem acquisition. Ticks, in contrast, are obligatory blood-feeders in all developmental stages, i.e. haemoglobin is a staple constituent of every tick diet. This means that ticks would not face a shortage of haem during feeding. Therefore, a signalling mechanism promoting *IrHRG* expression as a means to increase haem acquisition (negative regulation) cannot and does not exist. The tick *IrHRG* (HRG), is, in fact, likely haem-non-responsive. Having said that, we cannot achieve a zero haem diet, as we have not yet succeeded in feeding ticks on a chemically formulated diet that would allow omitting haemoglobin altogether, and thus we could not avoid having traces of haemoglobin in blood serum (accounting for 6–10 μM haem, electronic supplementary material, figure S6). The responsiveness of HRG to haem in unicellular parasites does not seem to be universal. The *Trypanosoma brucei* *tbr-hrg* homologue, which is mainly expressed in the insect stages rather than bloodstream forms infecting vertebrates, was not responsive to exogenous haem in the insect stages [12,13]. By contrast, the insect stage of another kinetoplastid parasite, *Leishmania amazonensis*, responds to haemin-replete conditions in the culture by suppressing mRNA levels of its HRG homologue [11], a similar mechanism to that of *C. elegans* [16]. An HRG homologue of *Trypanosoma cruzi* (*TcHRG*) responds to the presence of haem and decreases the transcript and protein level in the epimastigotes of the parasite [37]. It is noteworthy that experimental set-ups using diverse organisms with very complex life cycles cannot be compared and may not be aiming at the informative developmental stages where regulation presumably takes place, so might be over-looked or really be non-existent.

Expression of *IrHRG* in *hem1Δ* yeasts enhanced exogenous haem-mediated cellular proliferation (figure 3), similarly to HRGs from other metazoan parasites [14,15], as well as enhancing catalase activity. Supplementation of yeast medium with haemoglobin induced higher catalase activity than equimolar haem supplementation in HRG-expressing yeast cells (electronic supplementary material, figure S3). We speculate that protein-bound haem, in the form of haemoglobin, is more bioavailable as it is taken up by endocytosis and trafficked to the digestive vacuole where the liberated haem is then efficiently imported into the cytosol by highly abundant *IrHRG* at the organelle membrane. By contrast, haem not complexed with a protein vehicle might be prone to polymerization [38], decreasing its specific cellular uptake. Altogether, our yeast-based data supports the concept of *IrHRG* aiding translocation of exogenous haem across (sub)cellular membranes inside the cell.

RNAi-mediated silencing was employed to elucidate *IrHRG* function in ticks. Using light, fluorescent and electron microscopy, we visualized the accumulation of haem or haem analogue signals in vesicles of tick digest cells. Interestingly, we did not observe reduction of haemoglobin size upon RNAi as demonstrated for another haem transporter [39], but in fact the opposite, i.e. an increase of haemosome size. We thus speculate that haemosomes are formed either upon association with digestive vesicles or represent their terminal stage. One way or another, haem aggregates in vesicles where proteases are no longer present, indicating export from digestive vesicles. We argue that immediate diversion of liberated haem from the digestive vesicles into haemosomes prevents haem-mediated inhibition of acidic proteases, similar to a mechanism reported for *Plasmodium* spp. [40]. In contrast with ticks, however, haemozoin forms within the site of haemoglobin digestion, i.e. the digestive vacuole of *Plasmodium* spp. [41,42]. Our results further suggest that haem is expelled from digestive vesicles through physical association with destined haemosomes. Enlargement of haemosomes in *IrHRG*-KD ticks can be interpreted in a way that *IrHRG*, likely localized to the haemosome membrane, functions as regulatory vent controlling the release of haem to the cytosol. This hypothesis presents haemosomes as a sink of liberated haem, which is constantly imported from digestive vesicles, and then actively regulates cellular bioavailability of haem in midgut digest cells through *IrHRG*-mediated export, and retains excess haem as an aggregate within the haemosomes. Certainly, highly specific anti-*IrHRG* antibodies are needed to unambiguously confirm the protein localization. This will further help us disclose whether haem is released to the cytosol directly from the digestive vesicles, through haemosomes, or both.

From an applied point of view, blocking potential haem trafficking by *IrHRG* silencing did not result in reduced viability or developmental effects in blood-fed or low-haemoglobin serum-fed ticks (electronic supplementary material, figure S6). Loss-of-function (knock-downs/knock-outs) data from unicellular parasites mostly resulted in a compromised content of imported haem and reduced cellular viability. While the *hrg*-KD in the bloodstream form of *T. brucei* resulted in growth arrest and morphological alterations [12], the *hrg*-KD of *T. brucei* promastigotes did not result in any reduced growth phenotype under standard culture conditions unless challenged with low haemcin conditions; this significantly reduced the proliferation rate of the parasite [13]. *L. major* single knock-out promastigotes clearly contained lower levels of haem
or ZnPPIX compared to the control, and HRG add-backs restored the take-up levels of porphyrins [12]. L. amazonensis hrg-single knock-out parasites replicated more slowly in a haemin-poor medium [24]. Similarly to unicellular parasites [43], ticks code for haem transporters of different protein families. For example, the adenosine triphosphate (ATP)-binding cassette (ABC) transporter ABCB10, identified in R. microplus (GenBank accession number JN098464.1), was shown to be a haem transporter [39]. Unlike previously described haem transporters of the ABC protein family localized to mitochondrial membranes [44], the tick midgut ABCB10 is likely located in the membrane of the digestive vesicles. Its I. ricinus orthologue (GenBank accession number GAP1009746.1) [45] thus might compensate for reduced levels of HRG in respective haem transporting pathways. The lack of apparent feeding phenotype in IrHRG-KDs negates the potential of this protein to be an effective drug target [34], as it might do for unicellular parasites [1,46].

Enzymes or proteins that use haem as a cofactor may insert GaPPIX in their catalytic centres instead of haem, rendering the enzymes inactive. As gallium has only one valence state, Ga3+, it cannot be oxidized or reduced and is thus unable to substitute for Fe in biological reactions [47]. In Pseudomonas aeruginosa, GaPPIX inhibits its aerobic growth by targeting cytochromes, thus interfering with cellular respiration [48].

Blood-feeding of ticks, which likely saturates haem-binding proteins with acquired haem, thereby preventing GaPPIX from exerting its toxicity, allowed ticks to reach comparable engorged weights irrespective of the levels of GaPPIX in their blood meal. By contrast, serum feeding, which likely leaves from exerting its toxicity, allowed ticks to reach comparable levels of cytochromes, thus interfering with cellular respiration [48].

4. Material and methods

4.1. Tick maintenance and natural feeding

Adult I. ricinus ticks were collected in the forest near České Budějovice. Ticks were kept at 24°C and 95% humidity under a 15:9 h day/night regime. All laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic no. 246/1992 Sb., ethics approval no. 25/2018. The study was approved by the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (CAS) and Central Committee for Animal Welfare, Czech Republic (protocol no. 1/2015).

4.2. Tick membrane feeding

Membrane feeding of ticks was performed using a six-well plate format according to Krober and Guerin [49]. Whole bovine blood was collected in a local slaughter house and manually defibrillated. To obtain serum, whole blood samples were centrifuged at 2500g, 10 min, 4°C and the resulting supernatant was collected and centrifuged again at 10 000g, 10 min, 4°C. Red blood cells were received as a pellet and were washed three times in sterile phosphate-buffered saline (PBS). Fifteen ticks were placed in a feeding unit lined with a thin (approximately 120 µm for adults; approximately 80 µm for juveniles) silicone membrane. After 24 h, unattached or dead ticks were removed and an equal number of males were added to the attached females in the feeding unit of adult ticks. Diets were exchanged in a 12 h regime, with concomitant addition of 1 mM ATP and gentamicin (5 µg ml−1). For diet supplementation, bovine haemoglobin (Sigma H2500) or haem (Sigma H9039) were used. For membrane feeding experiments, the haem stock solution (5 mM haem dissolved in 100 mM NaOH) was diluted to 625 µM final concentration, establishing haem equimolarity with 1% haemoglobin (w/v). GaPPIX (Frontier Scientific P40167) was dissolved in 100 mM NaOH as a 5 mM stock.

4.3. Growth assay of alas− deficient yeast strain and catalase assay

IrHRG was amplified from tick tissue-specific cDNA using full-sequence specific primers (electronic supplementary material, table S2). The insert was further sub-cloned into destination vector: pDR ccdB-GFP-ura3 by Gateway™ LR Clonase™ II Enzyme mix (ThermoFisher Scientific 11791020). The cloning reaction was transformed into chemically competent DH5a E. coli cells (ThermoFisher Scientific), which were selectively grown on LBKAN plates. Miniprep plasmid preparations were then obtained from LBKAN liquid cultures to confirm the sequence of the insert.

S. cerevisiae BY4742 wild-type and hem1Δ (BY4742hem1Δ: BY4742 MAT α his3Δ1;leu2Δ0;lys2Δ0;ura3Δ0;hem1::LEU2) were grown at 30°C in YPD (yeast extract–peptone–dextrose) or appropriate synthetic complete (SC) medium supplemented with 250 µM ALA hydrochloride, haem (Sigma) or GaPPIX (Frontier Scientific). Cells were grown in YPD + ALA and were transformed with pDR experimental or control plasmid through the lithium acetate protocol [50], and transformants were selected on 2% w/v glucose SC−ura− + ALA plates.

Catalase activity was measured as described [51], but with some modifications. Briefly, yeast cells were cultured for 24 h without ALA to deplete haem and then incubated for 16 h in the absence or presence of either 0.5 µM bovine haemoglobin or 2 µM haem. Washed yeast cells were then adjusted to a A600 of 5 in 1 ml of H2O. Three hundred microlitres of 1% Triton X-100 and 300 µl of 30% H2O2 were then added. Catalase-generated oxygen bubbles were trapped by Triton X-100 and observed as a foam, the height of which was proportional to enzyme activity.

4.4. Fluorescent microscopy and image processing (yeast cells)

The haem-1Δ yeast strains expressing IrHRG, LmHRG, GFP or GPP were used in the exponential growth phase. Yeast cells were washed twice in 500 µl of PBS, fixed with 2%
paraformaldehyde and processed by microscopic observation. Images of yeast cells were acquired using an Olympus epifluorescence microscope and further processed using FIJI software (http://www.fiji.sc/Fiji).

4.5. Midgut micro-dissection, RNA extraction, cDNA synthesis and RT-qPCR

Membrane-fed *I. ricinus* larvae, nymphs and females were removed from the membrane or collected at the fully engorged state. Fully engorged larvae and nymphs were homogenized using a micro pestle in RA1 buffer (Macherey-Nagel, Germany) supplemented with β-mercaptoethanol. Midguts and ovaries of adult females were dissected under a drop of ice-cold DEPC-treated PBS and then homogenized in RA1 buffer supplemented with β-mercaptoethanol using a 29 G syringe. Total RNA was isolated from dissected tissues (intact or partial tissue from one individual was used per isolation) and whole body (seven larvae and a single nymph was used per isolation) homogenates using a NucleoSpinRNA II kit (Macherey-Nagel, Germany) and stored at −80°C. Prior to cDNA synthesis, RNA integrity was examined by agarose gel electrophoresis. cDNA preparations were made from 0.2 µg (juvenile ticks) or 0.5 µg (tissues of adult ticks) of total RNA in independent triplicates using the transcriptor high-fidelity cDNA synthesis kit (Roche Diagnostics, Germany). The cDNAs served as templates for subsequent quantitative expression analysis by RT-qPCR using the Fast Start Universal SYBR Green Master Kit (Roche). Samples were run on a LightCycler 480 (Roche) and relative expression was analysed by the ΔΔCt method. Expression profiles were normalized to *I. ricinus* elongation factor 1α (ef-1α) [52]. A list of primers is available as electronic supplementary material, table S2.

4.6. Phylogenetic analysis

The protein sequence obtained was verified by NCBI-BLAST search with the closest scores being to the *I. scapularis* and *A. variegatum* protein sequences. The dataset for following phylogenetic analysis was composed from available sequences in GenBank and UniProt to equally represent all the main groups of vertebrates, insects, nematodes and protists, among which the HRG transporter was identified. Sequences were aligned using the MAFFT software version 7 [53], and the final alignment was manually edited by Geneious Prime software 2019.1.1. (https://www.geneious.com). The best-fit model of molecular evolution was selected using aikake information criteria (AIC) in the ProtTest 3 software [54]. ML was performed under the empirical mixture model of substitution CAT-LG + G chosen by AIC. ML analyses were computed by the W-IQ-Tree software [55]. The reliability of branching patterns within the ML tree was tested by bootstrapping with 1000 resamplings, and the analyses were run independently and repeated three times. The final tree was visualized and rooted in the FigTree software v. 1.4.4 [56]. The protein (IHRG) sequence was deposited in GenBank/UniProt under accession number A0A131XS12. Nei’s genetic distances between sequences were calculated in Geneious.

4.7. ZnPPIX fluorescent microscopy

Uptake of a fluorescent metalloporphyrin, zinc mesoporphyrin, as a fluorescent haem analogue, was used to characterize haem intracellular pathways similarly to a previously published protocol [39]. A 20 mM stock solution of ZnPPIX (Sigma-Aldrich 691150-M) was dissolved in 100 mM NaOH and further diluted in sterile PBS before serving to ticks. Five microlitres of 100 µM ZnPPIX were introduced (2 h at 37°C) through 10 µl microcapillaries (Sigma-Aldrich P0674) into *I. ricinus* ticks, pre-fed 5 days on a guinea pig. Ticks were then rested for 24 h at 21°C. ZnPPIX was visualized in the dissected midguts of partially engorged ticks subjected as described below to RNA interference. The fluorescent images were obtained using a fluorescence microscope Olympus BX63 (filter U-PGWA, emission 575–625 nm) and DP74 digital camera.

4.8. Toluidine blue staining and transmission electron microscopy

Adult unfed females were injected with *IHRG* or *gfp* dsRNA (see below) and fed on guinea pigs for 5 days. The gut tissue was dissected and transferred to a fixative (2.5% glutaraldehyde) and incubated at 4°C, overnight. Samples were further washed with 0.1 M Na-phosphate buffer (PB) and incubated with 2% osmium tetroxide for 2 h at room temperature (RT), and washed again with PB. Tissues were dehydrated in ascending acetone dilutions (30%, 50%, 70%, 80%, 90%, 95% and 100% for 15 min at each step). The samples were infiltrated stepwise in acetone mixed with EMbed 812 epoxy resin (EMS) (acetone : epox ratios of 2 : 1, 1 : 1, 1 : 2, at RT, for 1.5 h at each step followed by 100% Epon resin overnight). Embedded samples were thermo-polymerized at 62°C for 48 h. Semi-thin sections (400 nm) were stained with toluidine blue for 1 min at 60°C.

Ultra-thin sections (90 nm) were picked up on copper grids, contrasted in ethanolic uranyl acetate for 30 min and in lead citrate for 20 min. Semi-thin sections were observed under the Olympus BX53 light microscope and ultra-thin sections were observed in a JEOL 1010 transmission electron microscope.

4.9. High-pressure freezing and immuno-gold labelling

Guts were dissected from semi-engorged ticks. A piece of gut was transferred to a copper carrier in 20% bovine serum albumin (BSA) in PBS and immediately inserted into the rapid transfer system of the EM PACT2 (Leica) and frozen at a pressure of 2.1 kbar. The frozen samples were transferred under liquid nitrogen to cryovials with anhydrous acetone containing 0.25% glutaraldehyde and 0.25% uranylacetate. For freeze substitution, cryovials were placed in the chamber of a Leica APS freeze substitution system/device (Leica EM AFS) precooled to −90°C. Samples were maintained 96 h at −90°C. Freeze-substituted samples were then allowed to warm to −20°C at a rate of 5°C/h. After 23 h were samples allowed to warm again at a rate of 5°C/h to −10°C. At −10°C were specimens washed three times for 1 h in fresh anhydrous acetone. After the washes, samples were gradually infiltrated with LRW resin (London Resin Company Ltd) diluted in acetone at −10°C (in ratios of 10% and 40% for 1 h and 60% and 80% for 2 h and in 100% resin for 2 h and overnight). After infiltration, LR white was polymerized under UV light for 48 h at −10°C in the FS unit. Ultra-thin sections (90 nm in thickness) were cut on a Leica ultramicrotome and placed on formvar-coated copper grids. Sections were blocked in 1% FSG (fish
skin gelatine) in PBS + 0.1 M glycine for 1 h and subsequently exposed to antigen-purified primary antibody (cathepsin B, 1: 5 in blocking solution) for 3 h. Grids were washed 3 × 1% FSG in PBS + 0.1 M glycine for 3 min and incubated with secondary antibody (goat anti-rabbit, 1: 40) conjugated to gold particles of 10 nm in diameter for 1 h. In the end, all sections were washed again several times with blocking solution and dH₂O. After immunolabelling, sections were contrasted in ethanolic uranyl acetate for 5 min, lead citrate for 3 min and observed in a JEOL 1010 transmission electron microscope.

4.10. RNA interference
dsRNA was designed as before [34], and synthesized using the MEGAscript T7 transcription kit (Ambion) according to the previously described protocol [34]. I. ricinus females were injected into the haemocoel through to the coxae with IHRG-specific dsRNA or control gfp dsRNA (0.4 µl; 3 µg µl⁻¹) using a Narishige microinjector, allowed to rest for 1 day and then fed naturally on guinea pigs or on the artificial membrane feeding system. The efficiency of RNA-mediated silencing of IHRG gene expression was verified by RT-qPCR in tick midguts (figure 5a) and ovaries (electronic supplementary material, figure S7). Fully engorged weights were recorded, and embryos in oviposited eggs were visualized by light microscopy identically to our previously established protocol [3].

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**Data accessibility.** This article has no additional data.

**Authors’ contributions.** J.P. designed the study, carried out molecular laboratory work and drafted the manuscript; T.H. performed tick membrane feeding experiments; M.C.D. assisted in yeast-based assays; V.U. performed light and fluorescent microscopy; D.S. participated in data analysis; H.F. performed TEM imaging; D.H. participated in data analysis; D.J. carried out the phylogenetic analysis; J.M.P.V., coordinated the yeast-based experiments and helped draft the manuscript; P.K. participated in data analysis and helped draft the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

**Competing interests.** We declare we have no competing interests.

**Funding.** This work was primarily funded by the Czech Science Foundation, grant no. 18-01832S and, additionally by grant no. 21-08826S. J.P. and P.K. are also grateful for financial support from the ‘Centre for research of pathogenicity and virulence of parasites’ (no. CZ.02.1.01/0.0/0.0/16_019/0000759) funded by the European Regional Development Fund (ERDF) and Ministry of Education, Youth and Sport (MEYS). We acknowledge the core facility Laboratory of Electron Microscopy supported by MEYS CR (LM2018129 Czech-Biomaging). The work was also supported by the Spanish Ministerio de Economía y Competitividad (PID2019-106724RB-I00) to J.M.P.V., Junta de Andalucía (P18-RT-3052 to J.M.P.V.) and FEDER funds from the EU to J.M.P.V.

**Acknowledgements.** The authors are grateful for technical assistance of Jan Erhart from the tick animal facility.

TORH is involved in the heme transport from the phagolysosome to the cytosol. The role of TORH in heme transport is essential for the survival of parasites in the host. In Leishmania species, TORH is regulated by the conserved and concerted functions of HRG-1 proteins. Nature 453, 1127–1131. (doi:10.1038/nature06934)
