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RESEARCH ARTICLE

Multiple ferritins are vital to successful blood feeding and reproduction of the hard tick *Haemaphysalis longicornis*

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SUMMARY

Ticks are obligate hematophagous parasites and important vectors of diseases. The large amount of blood they consume contains great quantities of iron, an essential but also toxic element. The function of ferritin, an iron storage protein, and iron metabolism in ticks need to be further elucidated. Here, we investigated the function a newly identified secreted ferritin from the hard tick *Haemaphysalis longicornis* (HlFER2), together with the previously identified intracellular ferritin (HlFER1). Recombinant ferritins, expressed in *Escherichia coli*, were used for anti-serum preparation and were also assayed for iron-binding activity. RT-PCR and western blot analyses of different organs and developmental stages of the tick during blood feeding were performed. The localization of ferritins in different organs was demonstrated through an indirect immunofluorescent antibody test. RNA interference (RNAi) was performed to evaluate the importance of ferritin in blood feeding and reproduction of ticks. The midgut was also examined after RNAi using light and transmission electron microscopy. RT-PCR showed differences in gene expression in some organs and developmental stages. Interestingly, only HlFER2 was detected in the ovary during oviposition and in the egg despite the low mRNA transcript. RNAi induced a reduction in post-blood meal body weight, high mortality and decreased fecundity. The expression of vitellogenin genes was affected by silencing of ferritin. Abnormalities in digestive cells, including disrupted microvilli, and alteration of digestive activity were also observed. Taken altogether, our results show that the iron storage and protective functions of ferritin are crucial to successful blood feeding and reproduction of *H. longicornis*.

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INTRODUCTION

Ticks are obligate blood-sucking parasites known to transmit a wide variety of infectious diseases worldwide. They are considered second to mosquitoes in terms of their impact on public health, but they are the most important vectors of different pathogens in both domestic and wild animals (Nicholson et al., 2009). Tick-borne diseases continue to have great economic impact on livestock production, particularly on cattle and small ruminants, in several continents (Jongejan and Uilenberg, 2004). The hard tick *Haemaphysalis longicornis* Neumann 1901 is widely distributed in East Asia and Australia, where it transmits theileriosis and babesiosis (Fujisaki et al., 1994; Jongejan and Uilenberg, 2004). Thus, tick control is essential in controlling tick-borne diseases. Different methods of tick control, including the use of chemical acaricides and vaccines, are presented with several challenges (Willadsen, 2006). Studies on the identification of tick protective antigens that can be used on the formulation of potent vaccines continue to gain much interest.

Hematophagous arthropods, including ticks and mosquitoes, utilize blood for nutrients and reproduction. The blood meal of female mosquitoes provides iron, which is required for optimal egg development and offspring viability (Zhou et al., 2007). In female ticks, initiation of the reproductive cycle necessitates a blood meal. Host-derived heme is bound to vitellogenin and further incorporated into developing oocytes (Donohue et al., 2009). Most hematophagous arthropods ingest enormous amounts of blood in a single meal (Graça-Souza et al., 2006). Adult hard ticks can ingest a large volume of blood, 200 to 600 times their unfed body weight (Rajput et al., 2006). This also exposes them to large amounts of iron.

Iron is both an essential and a toxic element to living organisms. Iron is important in most cells as a cofactor for fundamental biochemical activities including oxygen transport, energy metabolism and DNA synthesis. However, iron can also catalyze the propagation of reactive oxygen species and the generation of

highly reactive radicals (Wang and Pantopoulos, 2011). These radicals can attack and damage cellular macromolecules and promote cell death and tissue injury (Papanikolaou and Pantopoulos, 2005). To address this problem, different organisms utilize several proteins for iron metabolism.

Ferritin is an iron storage protein that plays an important role in the iron metabolism of most organisms. In general, ferritins consist of 24 subunits, which fold in a 4-helical bundle, with a large cavity that can accommodate up to 4000 Fe atoms (Arosio et al., 2009). Vertebrate ferritins are composed of two types of subunits, the heavy (H) chain, associated with Fe(II) oxidation, and the light (L) chain, which assists in iron nucleation and core formation (Harrison and Arosio, 1996). Similarly, insect ferritins have two types of subunits, referred to as a heavy-chain homolog (HCH) and a light-chain homolog (LCH) (Pham and Winzerling, 2010). Ferritin functions both in iron homeostasis and protection against oxidative damage. Ferritin is considered to be a critical cytoprotective protein that comprises an essential part of the antioxidant response (Tsuji et al., 2000). In insects, it also plays a role in iron transport (Pham and Winzerling, 2010).

In ticks, iron metabolism remains poorly understood. The hard tick *Rhipicephalus (Boophilus) microplus* lacks a heme synthetic pathway (Braz et al., 1999). A heme-binding lipoprotein, HeLp, has been described in this tick as a special adaptation. HeLp transports heme in the hemolymph and peripheral tissues, primarily the ovaries (Maya-Monteiro et al., 2000). The ferritin gene from different species of ticks (Kopáček et al., 2003; Xu et al., 2004) has already been identified. Knockdown of two ferritins from *Ixodes ricinus* caused adverse effects on tick feeding and reproduction (Hajdusek et al., 2009).

Here, we identified a new secretory ferritin from *H. longicornis*, ferritin 2 (HlFER2), and characterized it together with the previously reported intracellular ferritin, herein referred to as ferritin 1 (HlFER1). We examined the distribution and expression pattern of both ferritins in different tissues and developmental stages of the tick. We further evaluated the physiological importance of ferritin in the blood feeding and reproduction of the tick through a reverse genetic approach. Abnormal findings in the midgut after gene silencing further demonstrated the crucial function of ferritin as a safeguard against iron toxicity in the hard tick.

MATERIALS AND METHODS

Ticks and experimental animals

The parthenogenetic, Okayama strain of *H. longicornis* was used in all experiments throughout this study. Ticks were maintained by feeding on ears of Japanese white rabbits (Kyudo, Kumamoto, Japan) for several generations at the Laboratory of Emerging Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan (Fujisaki, 1978). Mice (strain ddY, 6 weeks old, female) were used for ferritin anti-serum preparation. The experimental animals were treated following the approved guidelines from Animal Care and Use Committee of Kagoshima University (Approval number A08010).

Identification and characterization of *Hlfer* cDNA clones

The full-length cDNA library of *H. longicornis* was constructed using the vector-capping method (Kato et al., 2005), and expressed sequence tags (EST) were prepared in our laboratory (Boldbaatar et al., 2010b). cDNA clones encoding ferritin were identified and selected from the EST database for further analysis. Plasmids containing *Hlfer* gene-encoding inserts were extracted using the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) and were subsequently analyzed using plasmid-specific primers through an automated sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) to determine the full-length sequences. The deduced amino acid translation of *Hlfer* genes was determined using GENETYX software (Genetyx, Tokyo, Japan). Homologous search of the full-length *Hlfer* sequences was performed using the BLAST program, through which putative conserved domains were also identified. The presence of signal peptide and its cleavage site was determined using the prediction server SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), and the predicted molecular weight and isoelectric points (pIs) were determined using the ExPASy server (http://web.expasy.org/peptide_mass/). Analyses for *N*-glycosylation, domain structure and ligand binding were performed using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), PROSITE software (http://prosite.expasy.org/), and PDBe motif and site software (http://www.ebi.ac.uk/pdbe-site/pdbemotif/), respectively. A phylogenetic tree was also constructed based on the amino acid sequences of ferritins from selected species by the neighbor-joining method using MEGA software version 5.0 (Tempe, AZ, USA).

Expression and purification of recombinant proteins

The open reading frame (ORF) of *Hlfer1* and *Hlfer2* was extracted from pGCAP1 vector using gene-specific primers. For *Hlfer2*, a forward primer without signal peptide sequence was used. PCR products were purified using the GENECLEAN II kit (MP Biomedicals, Solon, OH, USA) and then subcloned into the pRSET A vector (Invitrogen, Carlsbad, CA, USA). The resulting plasmids were checked for accurate insertion through restriction enzyme analysis and purified using the Qiagen Plasmid Mini Kit (Qiagen). Purified plasmids were expressed in *Escherichia coli* BL21 cells, grown in Luria–Bertani broth medium with ampicillin. Synthesis of recombinant ferritins tagged with histidine was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mmol l^{-1}. Cells were collected by centrifugation, and protein was extracted through ultra-sonication. Purification was carried out through chromatography using a HisTrap column (GE Healthcare, Uppsala, Sweden) and then dialysis against phosphate-buffered saline (PBS). Purity was checked by SDS-PAGE. Concentration of proteins was also determined through SDS-PAGE using bovine serum albumin as standard. The Micro BCA Assay kit (Thermo Scientific, Rockford, IL, USA) was also used for soluble proteins. Purified recombinant proteins were used to immunize mice for the production of antibodies.

Preparation of mouse anti-ferritin sera

To prepare mouse anti-ferritin sera, each mouse was injected intraperitoneally with 100 μg of recombinant ferritin and completely mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St Louis, MO, USA). Immunization was repeated 14 and 28 days after the first immunization, using an incomplete adjuvant (Sigma-Aldrich). All sera were collected 14 days after the last immunization.

Total RNA extraction and reverse-transcriptase PCR analysis

Total RNA was extracted from different developmental stages (egg, larva, nymph and adult) and tissues of adult female ticks, including the midgut, salivary glands, ovary, fat body and hemocytes, during blood feeding. For different developmental stages, whole tick samples were homogenized using a mortar and pestle in TRIzol reagent (Invitrogen). Tissues were dissected and washed in PBS and then placed directly in tubes with the TRIzol reagent. Hemocytes

were collected as described previously (Aung et al., 2011; Fujisaki et al., 1975). RNA extraction was performed based on the manufacturer's protocol. Single-strand cDNA was prepared by reverse transcription using the Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) following the manufacturer's protocol. PCR was carried out using ferritin gene-specific primers (supplementary material Table S1). Primers for actin, used for control amplification, and vitellogenin genes are described elsewhere (Boldbaatar et al., 2010a). PCR products were subjected to electrophoresis in 1.5% agarose gel in a TAE buffer, and bands were visualized after staining the gel with ethidium bromide using Quantity One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy).

Protein extraction and western blot analysis

Protein extraction was performed as described previously (Aung et al., 2011; Boldbaatar et al., 2006). Tick protein extracts were separated with 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked overnight with 5% skim milk in PBS with 0.05% Tween 20 and then incubated with a primary antibody using mouse anti-ferritin sera (1:500 dilution). For detection of protein in the whole and different tissues of adult ticks, an antiserum previously prepared for β-actin (Liao et al., 2008) was used as the control. Alternatively, β-tubulin (Umemiya-Shirafuji et al., 2012) antiserum was used for experiments that include the egg, wherein actin band was difficult to detect, particularly in the early stage of embryogenesis. After incubation with peroxide-conjugated sheep anti-mouse IgG (1:50,000 dilution; GE Healthcare, Little Chalfont, Buckinghamshire, UK), a signal was detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and analyzed using FluorChem FC2 software (Cell Biosciences, Santa Clara, CA, USA).

Indirect immunofluorescent antibody test

The indirect immunofluorescent antibody test (IFAT) was performed to demonstrate the endogenous localization of *H. longicornis* ferritins, following the method previously described (Aung et al., 2011; Umemiya et al., 2007). Briefly, the midgut, salivary glands and ovaries from 4-day partially fed adult ticks were fixed overnight in 4% paraformaldehyde in PBS with 0.1% glutaraldehyde added, and then washed with different concentrations of sucrose in PBS. The organs were embedded in Tissue-Tek OCT Compound (Sakura Finetek Japan, Tokyo, Japan) and then frozen using liquid nitrogen. Tissue sections of 10 µm thickness were cut using a cryostat (Leica CM 3050; Leica Microsystems, Wetzlar, Germany) and placed on MAS-coated glass slides (Matsunami Glass, Osaka, Japan). After overnight blocking with 5% skim milk in PBS at 4°C, sections were incubated with a 1:50 dilution of anti-ferritin sera for 2 h at room temperature. Normal mouse serum was used as a negative control at the same dilution. Sections were washed with PBS and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000; Invitrogen) for 1 h at room temperature. Following washes with PBS, the tissue sections were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken using a fluorescence microscope mounted with a DP71 camera and processed using DP Controller software (Olympus, Tokyo, Japan).

RNA interference (RNAi)

The PCR primers used for the synthesis of double-stranded RNA (dsRNA) are listed in supplementary material Table S1. The *Hlfer* fragments were amplified by PCR from cDNA clones using oligonucleotides, including T7-forward and T7-reverse primers, to attach the T7 promoter recognition sites on both the forward and reverse ends. The firefly *luciferase* (*Luc*) was amplified from a vector DNA of pGEM-luc (Promega, Madison, WI, USA) through PCR using oligonucleotides containing T7-forward and T7-reverse primers. PCR products were purified using the GENECLEAN II kit (MP Biochemicals). The T7 RiboMAX Express RNA System (Promega) was used to synthesize dsRNA by *in vitro* transcription following the manufacturer's protocol. Successful construction of dsRNA was confirmed by running 1 µl of the dsRNA products in a 1.5% agarose gel in a TAE buffer. Microinjection of dsRNA was performed as previously described (Aung et al., 2011; Boldbaatar et al., 2006). Briefly, 1 µg of *Hlfer1* or *Hlfer2* dsRNA in 0.5 µl of PBS was injected into the hemocoel of unfed adult female ticks through the fourth coxae. *Luc* dsRNA was injected in the control group. A total of 40 ticks per group were injected. After injection, the ticks were held for 18 h in a 25°C incubator to check for mortality resulting from possible injury during injection. Injected ticks from both the experimental and control groups were simultaneously infested on rabbit ears, with one group in each ear. To confirm gene-specific silencing, 10 ticks from each group were collected 4 days after attachment, and then total RNA and protein lysates were prepared for RT-PCR and western blot analysis, respectively. The remaining ticks were allowed to feed to repletion until they naturally dropped off from the host. The success of tick feeding was determined by measuring the weight and mortality after detachment from the host. The success of reproduction was determined by oviposition and hatching of eggs. This experiment was performed four times.

Histological and ultrastructural characterization of the midgut after RNAi

For the histological analysis of the midgut, 4-day partially fed ticks from *Luc* and *Hlfer* dsRNA-injected groups were collected and fixed overnight in 10% formalin and then embedded in paraffin. After deparaffinization and rehydration, sections were stained with hematoxylin & eosin (HE), mounted and observed under the microscope. Images were taken using a microscope mounted with a DP71 camera (Olympus).

For transmission electron microscopy (TEM), sections were prepared as previously described (Matsuo et al., 2003). Briefly, midgut samples were fixed in cold 3% glutaraldehyde in a sodium cacodylate buffer followed by post-fixation with 1% OsO_4 in the same buffer for approximately 2 h. After dehydration with an ethanol series, fixed samples were embedded in epon resin, and sections were cut using a Leica UCT ultramicrotome (Leica Microsystems) with a diamond knife. Sections were observed under a Hitachi H7000KU electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Iron-binding activity of recombinant HlFER2

The iron-binding activity of recombinant HlFER2 was determined and compared with that of commercially prepared horse apoferritin (MP Biomedicals LLC) using the ferrozine-based assay (Riemer et al., 2004; Zheng et al., 2010). The recombinant HlFER2 and horse apoferritin were dissolved in 1 ml water and mixed with 20 µl of 2 mmol l^{-1} $FeCl_2$ to reach final concentrations ranging from 75 to 600 nmol l^{-1}. Another *His*-tagged protein purified in our laboratory, the recombinant short C-type mannose receptor homolog (HlMRC) (H.M., K.F. and T.T., unpublished results), was used as negative control. After incubation at 30°C for 30 min, 40 µl of 6 mmol l^{-1} ferrozine (Sigma-Aldrich) was added and incubated further for

30 min with constant shaking. Each mixture was transferred to three wells in a microtiter plate, placing 300 µl in each well, and absorbance was measured at 550 nm using a microplate reader (Bio-Rad). The average absorbance reading was obtained, and the experiment was performed four times. The recombinant HlFER2 and horse apoferritin were also subjected to 12% SDS-PAGE and 3–10% native gradient polyacrylamide gels (Pagel, ATTO, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using Student's *t*-tests, with significant difference defined by $P<0.05$.

RESULTS

Identification and characterization of ferritin cDNAs

In this study, two full-length cDNAs encoding ferritin were identified and cloned from the EST database of *H. longicornis*. The full-length *Hlfer1* we identified contains 814 bp, with the predicted start codon at 142–144 bases, the stop codon at 664–666 bases, and an ORF extending from position 142 to 666, encoding for 174 amino acid polypeptides (supplementary material Fig. S1A). In agreement with a previous report, the 5′ untranslated region (UTR) of *Hlfer1* contains a putative iron-responsive element (IRE), with the highly conserved CAGUGA loop (Xu et al., 2004). The calculated molecular mass based on this sequence is ~20 kDa and has a pI of 4.97. *N*-glycosylation site prediction analysis of HlFER1 revealed a potential *N*-glycosylation site at position 109 NQS. Meanwhile, *Hlfer2* has 1635 bp, with the predicted start codon at 43–45 bases, the stop codon at 640–642 bases, and an ORF extending from position 43 to 642, coding for 199 amino acid polypeptides (supplementary material Fig. S1B). The nucleotide sequence data of *Hlfer2* have been deposited in the DDJB/EMBL/GenBank database under accession number AB734098. Unlike *Hlfer1*, *Hlfer2* does not have IRE in its 5′ UTR. A signal peptide was identified from the HlFER2 sequence using SignalP 3.0 Server prediction software, and a cleavage site was predicted between positions 17 and 18. The calculated molecular mass of HlFER2 without the signal peptide is ~20.8 kDa and has a pI of 5.29. No *N*-glycosylation site was identified in HlFER2. A ferritin-like diiron domain was identified between 30 and 180 amino acids. Ligand-binding statistics showed that both HlFER1 and HlFER2 have a high tendency for iron-binding. Both ferritin nucleotide sequences end with a 20 bp polyadenylation tail at the 3′ UTR.

BLAST analysis showed that HlFER1 has 100% homology with the previously identified *H. longicornis* ferritin (GenBank Accession number AY277905). It also showed high homology with ferritin of other hard ticks and the soft tick *Ornithodoros moubata*, while HlFER2 showed high homology with secreted ferritin of *I. ricinus*. Analysis of putative conserved domains showed that both ferritins contain the seven conserved amino acid residues in the ferroxidase center loop, which are important for metal binding, and a conserved ferrihydrite nucleation center, where ferric atoms are stored (Harrison and Arosio, 1996). Multiple alignment of amino acid sequences of ferritins from different ticks and selected species (supplementary material Fig. S2) demonstrated that HlFER1 has high identity with intracellular ferritin of the ticks *Rhipicephalus sanguineus* (AY277907.1), *O. moubata* (AF068225.2) and *I. ricinus* (AF068224.1), followed by the human ferritin H-chain (NM_002032.2), *Drosophila melanogaster* ferritin1 HCH (AF145125.1) and the *Aedes aegypti* putative ferritin subunit (XM_001654469.1). In contrast, HlFER2 shares high identity with *I. ricinus*-secreted ferritin (EU885951.1), followed by intracellular ferritin of the ticks *R. sanguineus*, *O. moubata* and *I. ricinus*, the human ferritin H-chain, *D. melanogaster* ferritin1 HCH and the *A. aegypti* ferritin subunit. A phylogenetic tree was also constructed using amino acid sequences of ferritins from different species by the neighbor-joining method (supplementary material Fig. S3). HlFER1 was found to be closely related to intracellular ferritin of *I. ricinus*, whereas HlFER2 was found to be closely related to *S. mansoni* ferritin (XM_002576034.1).

Expression profile of ferritin transcripts from different tick organs and developmental stages

RT-PCR was performed to determine the expression profile of *Hlfer1* and *Hlfer2* from different organs of adult female ticks (Fig. 1) and different developmental stages of the tick during blood feeding (Fig. 2A). The organs included midgut, salivary glands, hemocytes, fat body and ovary. The amount of cDNA was indexed based on control amplification using actin-specific primers. Results showed that *Hlfer1* was constitutively expressed in all five organs during blood feeding as well as in different developmental stages. Whereas *Hlfer2* was also constitutively expressed in the midgut and hemocytes, decreasing expression was observed in the fat body and the salivary glands towards engorgement, and limited expression was observed in the ovary only in unfed ticks and on the first day of feeding. *Hlfer2* expression was strong in unfed and engorged larvae, nymphs and adults, weak in the partially fed stages, and barely detectable in the egg.

Detection of native tick protein using anti-ferritin sera

The expression of endogenous HlFER in different organs of adult female tick and different developmental stages was determined through western blot analysis using specific anti-ferritin sera. In different developmental stages, HlFER1 and HlFER2 were detected in partially fed and engorged but not in unfed larvae, nymphs and adults (Fig. 2B). Interestingly, HlFER2 was detected in the egg, but HlFER1 was not. A similar pattern of expression of HlFER1 and HlFER2 was observed in the midgut and salivary gland (Fig. 3A). In the midgut, HlFER expressions remained unchanged regardless of the degree of feeding. In salivary glands, the expressions increased during blood feeding. Meanwhile, in the hemolymph including hemocytes from partially fed adults, HlFER2 but not HlFER1 was detected (Fig. 3A). To evaluate the importance of HlFER during oviposition and embryonic development, protein

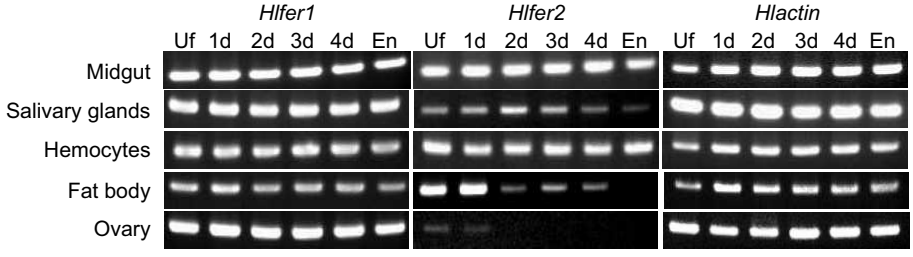

Fig. 1. Transcription profiles of *Hlfer1* and *Hlfer2* in different tissues of adult female *Haemaphysalis longicornis* during blood feeding. Gene-specific primers are indicated on the topmost row. Actin was used as a loading control. Uf, unfed; 1d, 1-day fed; 2d, 2-day fed; 3d, 3-day fed; 4d, 4-day fed; En, engorged tick.

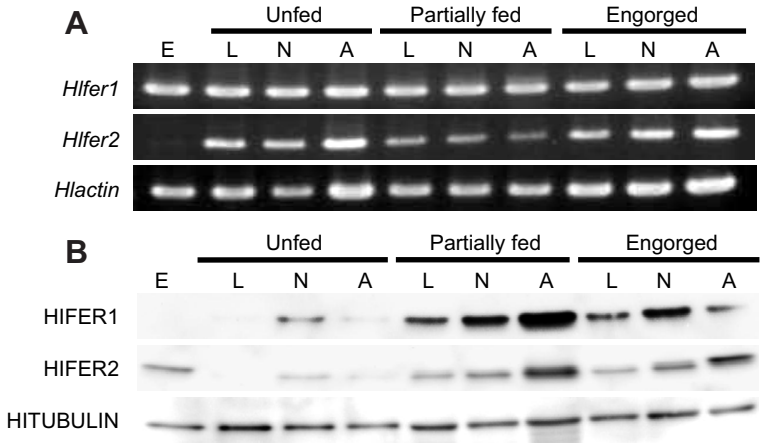

Fig. 2. RT-PCR (A) and western blot (B) analyses in different developmental stages of *H. longicornis* using ferritin-specific primers and mouse sera. Total RNA or protein lysates were prepared from the egg and whole nymphs, larvae and adults in different states of feeding (unfed, partially fed and engorged). Actin primer and mouse anti-tubulin serum were used as a loading control for RT-PCR and western blot analysis, respectively. E, egg; L, larva; N, nymph; A, adult.

expression was checked in the ovary at different times during oviposition (Fig. 3B) and in the eggs at different stages of embryonic development and immediately after hatching (Fig. 3C). HlFER1 showed strong expression only in unfed ovary but none in the egg at any stage of development (data not shown). HlFER2, meanwhile, was expressed in unfed ovary, and expression decreased during feeding but increased again during the oviposition period. In the egg, HlFER2 was constitutively expressed throughout embryonic development and decreased in newly hatched larvae.

Demonstration of HIFER in different organs using IFAT

The endogenous localization of HlFER1 and HlFER2 in adult midgut, salivary glands and ovary was demonstrated using IFAT

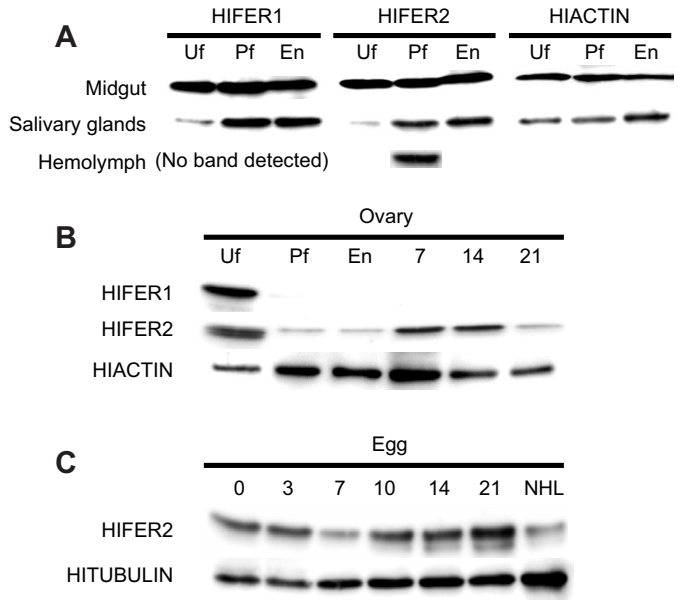

Fig. 3. Western blot analyses for HlFER1 and HlFER2 using specific mouse anti-HlFER sera. Actin or tubulin antiserum was used as a control. (A) Protein expression in the midgut and salivary glands from unfed (Uf), 4-day partially fed (Pf) and engorged (En) adult ticks and hemolymph including hemocytes from partially fed adult ticks. (B) Protein expression in the ovary during feeding and oviposition. The numbers represent the days after engorgement, also corresponding to the oviposition period of the tick. (C) Protein expression in the egg during embryonic development and in newly hatched larva (NHL). The numbers represent the days after which the eggs were laid.

(Fig. 4). Both ferritins were found in the cytoplasm of digestive cells in the midgut. However, HlFER1 showed stronger fluorescence than HlFER2, which showed punctate fluorescence in the cytoplasm of digestive cells, suggesting that HlFER2 is located inside granules within the cells. In salivary glands, both HlFER1 and HlFER2 showed a dot-like pattern of fluorescence along the basement membrane of salivary acini and salivary duct. In the ovary, HlFER1 fluorescence was distributed throughout the oviduct and, to some extent, in the oocytes, whereas HlFER2 showed strong fluorescence on the surface of oocytes.

Impact of *Hlfer* silencing on feeding, survival and reproduction

To evaluate the importance of ferritins on blood feeding and reproduction of ticks, gene silencing through RNAi was performed. Ticks were individually injected with either *Hlfer1* or *Hlfer2* dsRNA or with *Luc* dsRNA for the control group. Silencing of both *Hlfer1* and *Hlfer2* had significant effects on feeding and fecundity, as shown in Fig. 5. The average body weight of both *Hlfer1*- and *Hlfer2*-silenced ticks was 72.1 and 63.6% lower than that of the control *Luc* group, respectively (Table 1). Clearly, silencing of both *Hlfer1* and *Hlfer2* caused failure to feed to repletion. *Hlfer*-silenced ticks showed high mortality after a blood meal, the *Hlfer1*-silenced group having more than 90% mortality. Furthermore, most ticks died without laying eggs. The control group did not have any mortality until the oviposition period had finished. *Hlfer* silencing also significantly reduced the fecundity of ticks. *Hlfer2*-silenced ticks that laid eggs had lower egg weight to body weight ratios, and the eggs had abnormal morphology and lower hatchability. Gene silencing from whole ticks and different organs was confirmed to be successful using RT-PCR, western blotting and IFAT (Fig. 6, supplementary material Figs S4, S5). Interestingly, we found that silencing of *Hlfer1* led to a reduction of HlFER2 expression in whole ticks and the midgut and its absence in the salivary glands and ovary, whereas silencing of *Hlfer2* reduced the expression of HlFER1 in the salivary glands and ovary.

To clarify the decreased reproductive capacity after silencing of *Hlfer1* and *Hlfer2*, we checked to determine whether there was an effect on the expression of different vitellogenin genes (*HlVg*) using RT-PCR (Fig. 7). Three vitellogenin genes (*HlVg-1*, *HlVg-2* and *HlVg-3*) have been identified from *H. longicornis* and have been shown to be essential for the tick's reproduction (Boldbaatar et al., 2010b). Interestingly, we found that silencing of *Hlfer1* led to silencing of *HlVg-1* and reduced the expression of *HlVg-3*, whereas

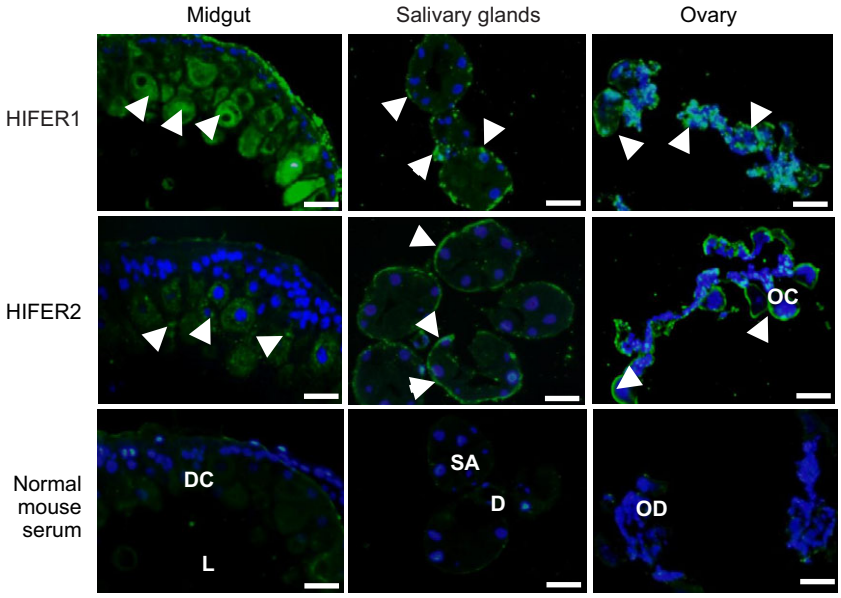

Fig. 4. Indirect fluorescent antibody test (IFAT) for the localization of endogenous HlFER1 and HlFER2 in partially fed midgut, salivary glands and ovary. Frozen sections were incubated with specific mouse anti-HlFER sera as primary antibodies. Normal mouse serum was used as a control. Mouse anti-IgG conjugated with Alexa 488 was used as a second antibody. Arrowheads point to positive fluorescence. DC, digestive cells; L, lumen; SA, salivary gland acinus; D, duct; OC, oocyte; OD, oviduct. Scale bars, 50 µm.

silencing of *Hlfer2* reduced the expression of both *HlVg-1* and *HlVg-3*. No effect on *HlVg-2* was observed from silencing of either *Hlfer1* or *Hlfer2*. Due to the marked effect of *Hlfer1* silencing on *HlVg-1*, we further examined its expression in the midgut, where it was reported to be mainly expressed (Boldbaatar et al., 2010b). Nevertheless, we only found reduction in expression after *Hlfer1* knockdown, in contrast with the result using whole tick cDNA (supplementary material Fig. S4C).

Microscopic analyses of the midgut after RNAi

To understand the effect of *Hlfer* knockdown on blood feeding, we performed histological and ultrastructural examination of the midgut. Morphological characteristics of midgut cells were observed based on previous reports (Agbede and Kemp, 1985; Agbede, 1986; Agyei and Runham, 1995). In ticks, blood meal components are phagocytosed, and digestion occurs inside digestive cells (Tarnowski and Coons, 1989). Based on HE sections and TEM of partially fed midgut, knockdown of either *Hlfer1* or *Hlfer2* resulted in abnormal morphology of digestive cells and apparently reduced their digestive activity (Fig. 8). HE sections revealed digestive cells with altered shape, vacuolated cytoplasm, and disrupted microvilli and cell membranes. These abnormalities were more pronounced in midguts of *Hlfer1*-silenced ticks (Fig. 8B). Eosinophilic granules were also observed in the cytoplasm of digestive cells, which may indicate cell degeneration. Diminished production of hematin was observed in HE sections, particularly from *Hlfer1*-silenced ticks. From TEM, fewer digestive cells containing hematin granules were observed in *Hlfer1*-silenced ticks, in contrast to control ticks. Furthermore, the microvilli of digest cells were short and sparsely distributed in *Hlfer1*-silenced ticks (Fig. 8E). No significant difference was observed between control and *Hlfer2* midgut sections (data not shown).

Iron-binding activity of recombinant HlFER2

A colorimetric assay using ferrozine as the iron-detecting reagent was used to determine whether the recombinant HlFER2 purified from *E. coli* has iron-binding activity. The absorbance reading is directly proportional to the available ferrous ion in the solution that reacted with ferrozine. As shown in Fig. 9A, the absorbance reading decreased with increasing concentration of both recombinant HlFER2 and horse apoferritin compared with the recombinant short HlMRC ($P<0.05$) used as a negative control, which did not show iron-binding activity. The recombinant HlFER2 displayed a sharp

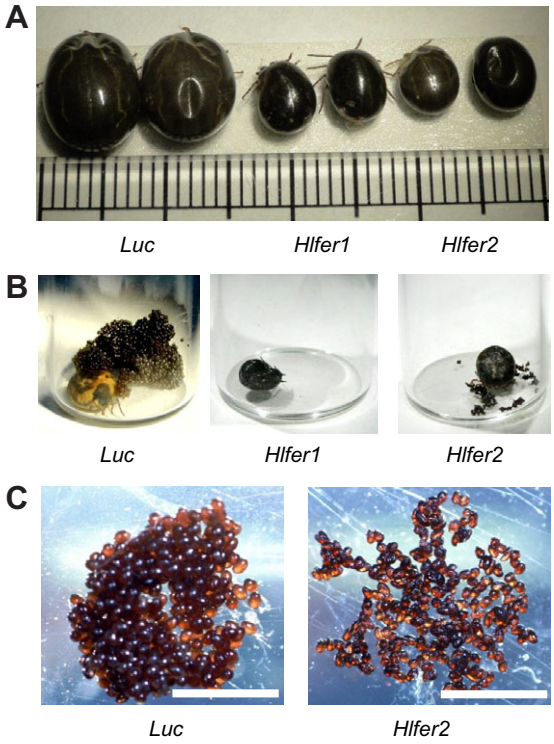

Fig. 5. Effects of RNAi-mediated silencing of *Hlfer* on the feeding and reproduction of *H. longicornis*. *Hlfer1* or *Hlfer2* dsRNA was injected into female ticks. *Luciferase* dsRNA was injected into the control group. Ticks were allowed to feed and naturally drop off from the host. (A) Both *Hlfer1* and *Hlfer2* dsRNA-injected ticks were smaller than the control upon detachment due to failure to fully engorge. (B) Mortality was high during oviposition for both *Hlfer* dsRNA-injected groups. Most *Hlfer1* dsRNA-injected ticks died without laying eggs, while some *Hlfer2* dsRNA-injected ticks laid very few eggs. (C) Eggs were examined under a steromicroscope. Eggs from *Hlfer2* dsRNA-injected ticks have an abnormal morphology. Scale bars, 1 mm.

Table 1. Effects of *Hlfer1* and *Hlfer2* silencing on blood feeding and reproduction of *Haemaphysalis longicornis*

Group	Body weight of ticks post-detachment (mg)	Mortality of ticks post-detachment (%)[a]	Egg weight/body weight (%)[b]	Percentage of ticks that laid eggs	Hatched eggs
Luc (control)	259.3±52.3	0	51.6±6.9	100	25 of 25
Hlfer1 dsRNA	72.5±34.7*	91.67	55	4.16*	1 of 1
Hlfer2 dsRNA	94.5±22.8*	54.54	40.3±0.59	36.36*	1 of 9

[a]Ratio of ticks that died to the total number of ticks until 20 days after ticks dropped off from the host.
[b]Mean ± s.d. calculated from ticks that laid eggs. For the *Hlfer1* dsRNA group, only one tick laid eggs.
*Significantly different *versus* control (P<0.05) as calculated by the Student's t-test.

decrease in absorbance at 75 nmol l^{-1}, but this decrease became smaller at 150 nmol l^{-1}. These results suggested that recombinant HlFER2 has iron-binding activity similar to that of horse apoferritin and that the *His*-tag did not induce this activity. Based on SDS-PAGE (Fig. 9B), the purified *His*-tagged recombinant HlFER2 has only one subunit, with a molecular weight of ~26 kDa, as compared with horse apoferritin with H- and L-subunits, with a molecular weight of ~20 and 19 kDa, respectively. With regards to native PAGE analysis (Fig. 9C), the recombinant HlFER2 does not form the typical 24-mer folding of most ferritins, in contrast to horse apoferritin.

DISCUSSION

Ticks rely completely on host blood for survival and reproduction. They consume great amounts of blood, which exposes them to potentially toxic levels of iron. Hence, ticks must have a protective mechanism against the detrimental effects of iron overload. Iron from the blood meal may be in the form of heme or host-transferrin-bound iron. Excessive heme resulting from hemoglobin degradation aggregates in specialized organelles called hemosomes within the digestive cells of the midgut as a detoxification mechanism (Lara et al., 2003). In the hard tick *H. longicornis*, intracellular ferritin has been previously identified, but its function has not been described (Xu et al., 2004). Here, we report on a new secretory ferritin from *H. longicornis*, HlFER2, and characterize it with the intracellular ferritin, HlFER1. Through RNAi, we show the importance of both ferritins in successful tick feeding and reproduction.

Sequence analysis showed that *Hlfer2* mRNA lacks an IRE, similar to the secretory ferritin of *I. ricinus* (Hajdusek et al., 2009), the yolk ferritin of *L. stagnalis* (von Darl et al., 1994), *S. mansoni* ferritins (Schüssler et al., 1996) and *Haliotis discus discus Abf1* (De Zoysa and Lee, 2007). In contrast to HlFER1, HlFER2 contains a signal peptide and was detected in the hemolymph using a specific antiserum. Insect ferritins are synthesized with a signal peptide (Pham and Winzerling, 2010) that accumulates in the vacuolar system and the secretory pathway, except in some insect species in which cytosolic ferritin also occurs (Nichol and Locke, 1990). IFAT demonstrated punctate distribution of HlFER2 in the cytoplasm of digestive cells, suggesting that HlFER2 is located inside granules and possibly within the secretory pathway. Further examination is necessary to confirm this observation.

The difference in mRNA and protein expression patterns of the two ferritins reflects the difference in regulatory mechanism. *Hlfer1* is regulated by the interaction of IRE with iron-regulatory protein (IRP), demonstrated by the absence of protein bands despite the presence of mRNA transcripts. IRE-IRP binding is known to regulate iron-binding proteins in mammals, controlling intracellular iron homeostasis (Thomson et al., 1999; Wang and Pantopoulos, 2011). IRE-IRP binding has also been reported in the translational regulation of mosquito HCH (Geiser et al., 2006). In contrast, *Hlfer2* seems to be transcriptionally regulated. Interestingly, HlFER2 was detected in the salivary glands of engorged ticks and the egg despite the very low mRNA transcript level. This suggests that HlFER2 is synthesized elsewhere in the adult. Based on gene and protein

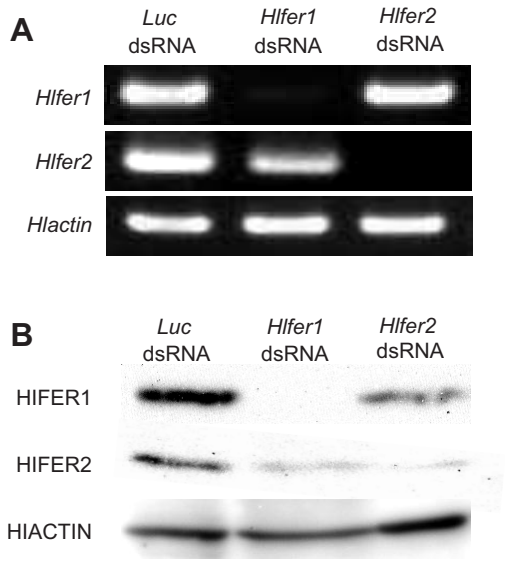

Fig. 6. Confirmation of *Hlfer* silencing. (A) Total RNA was extracted from whole 4-day fed ticks, and cDNA was subjected to RT-PCR using *Hlfer*-specific primers. (B) Western blot analysis of protein lysates prepared from whole 4-day partially fed ticks. Bands were detected using specific mouse anti-HIFER sera. Actin was used as a loading control.

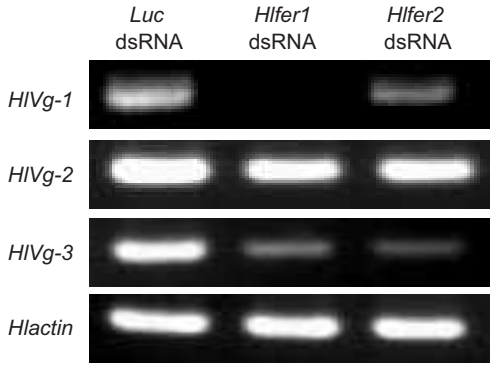

Fig. 7. Effect of *Hlfer* silencing on the expression of vitellogenin genes. cDNA from whole 4-day fed ticks injected with either *Hlfer1* dsRNA or *Hlfer2* dsRNA or with *Luciferase* dsRNA for the control group was amplified by three sets of primers of *HlVg-1*, *HlVg-2* and *HlVg-3*. The actin primer was used as a loading control.

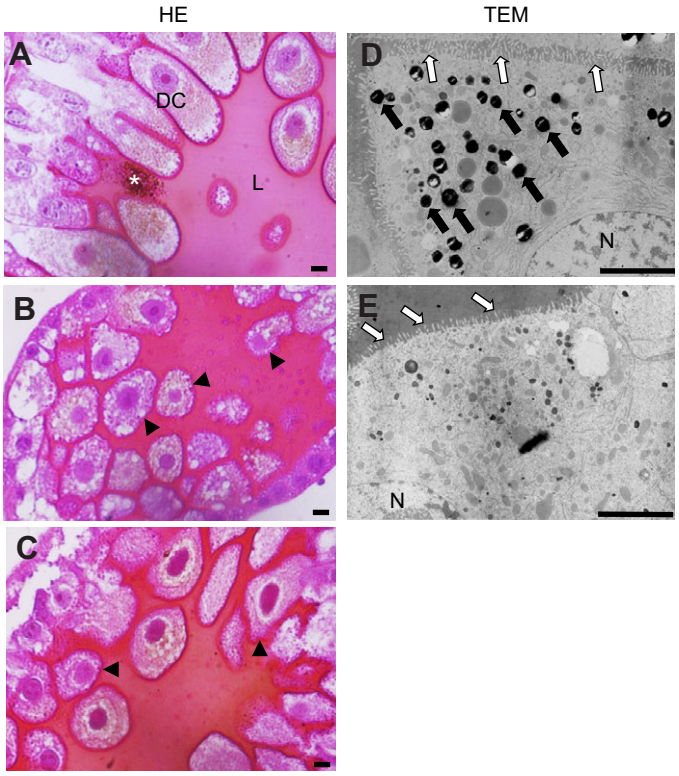

Fig. 8. Histological and ultrastructural examination of the midgut after knockdown of *Hlfer1* and *Hlfer2*. Hematoxylin & eosin (HE) staining of paraffin sections from *Luciferase* (A), *Hlfer1* (B) and *Hlfer2* (C) dsRNA-injected ticks was performed. Arrowheads point to disrupted microvilli and cell membrane. An asterisk indicates hematin granules released in the lumen. Transmission electron microscopy (TEM) micrographs of midgut from *Luciferase* (D) and *Hlfer1* (E) dsRNA-injected ticks are also shown. Black arrows point to hematin granules within the cell. White arrows point to microvilli. DC, digestive cells; L, lumen; N, nucleus. Scale bars, (A–C) 10 μm, (D,E) 0.5 μm.

expression, the midgut is the source of HlFER2, from which it is secreted to the hemolymph for transport to other organs, including maturing oocytes, and later incorporated in the eggs upon laying. This was further supported by the presence of HlFER2 in the ovary during the oviposition period and the strong fluorescence in the surface of oocytes revealed by IFAT. Moreover, the difference in the pattern of gene and protein expression of the two ferritins among organs may indicate variation in the role of these organs in iron metabolism. The midgut, being the organ involved in blood digestion and the first to be exposed to large amounts of iron, would also be the major organ for iron metabolism. The expression of both ferritins was unchanged from unfed to engorged midgut. IFAT also revealed the abundance of both ferritins in the cytoplasm of digestive cells.

Genetic manipulation through RNAi has been shown to be a very useful method in assessing gene function in ticks and a valuable tool for the characterization of tick protective antigens (de la Fuente et al., 2005; de la Fuente et al., 2007). In the present study, knockdown of both ferritin genes reduced the capacity of the ticks to fully engorge, and greatly impaired reproductive capacity. A study has been previously conducted in the ixodid tick *I. ricinus*, wherein knockdown of two ferritins affected feeding and reproduction of this hard tick (Hajdusek et al., 2009). Here we also showed that knockdown of both ferritins reduced the feeding capacity of *H. longicornis*. *Hlfer* RNAi could have altered iron homeostasis and resulted in iron overload. In the midgut, fewer digestive cells containing hematin were observed in HE sections and TEM after *Hlfer* knockdown. Hematin is the most prominent end-product of intracellular blood digestion, particularly of hemoglobin (Agbede and Kemp, 1985). This reduction was marked in the *Hlfer1*-silenced group, suggesting decreased digestive activity. Disrupted microvilli were also noted, which could have impaired the phagocytic activity of digestive cells. Microvilli increase the cell surface area and absorptive capacity of digestive cells (Agbede, 1986) and are possibly involved in receptor-mediated endocytosis (Balashov and Raikhel, 1976). Silencing of *Hlfer1* prevented the intracellular storage of iron in digestive cells, whereas silencing of *Hlfer2* prevented iron sequestration for delivery to other organs, including developing oocytes. In both cases, accumulation of iron within digestive cells decreased both the uptake and digestion of blood. Thus, ticks were unable to take up large amounts of blood and feed to repletion.

Ferritin responds to oxidative stress in mammalian cells (Orino et al., 2001) and functions in preventing oxidative challenge and iron toxicity in hematophagous insects (Geiser et al., 2003). High mortality after silencing of ferritin genes can be attributed to iron-induced cytotoxicity and oxidative stress. Iron is known to induce lipid peroxidation (Braughler et al., 1986; Fuhrman et al., 1994). Iron toxicity is largely due to its ability to catalyze the Fenton reaction, which leads to the generation of radicals that attack and damage cellular macromolecules and promote cell death and tissue injury (Papanikolaou and Pantopoulos, 2005). Abnormal morphologic features observed in HE sections of the midgut, particularly from *Hlfer1*-silenced ticks, suggested possible cell damage. We could not confirm these abnormal morphologic features found in HE sections through TEM, but decreased phagocytic and digestive activity was evident.

We hypothesized that HlFER1 is the primary ferritin in the midgut cells responsible for iron storage and that it interacts with HlFER2. This was confirmed by the results of western blot analysis after *Hlfer1* silencing, wherein HlFER2 expression was reduced in the midgut and abolished in salivary glands and ovary. These data suggest that HlFER2 secretion from the midgut might be affected by HlFER1 expression. Moreover, *Hlfer1* silencing had more distinct effects on midgut cell morphology and digestive activity, implying a greater role in protection against iron toxicity. A previous study on *I. ricinus* also showed that silencing of *fer2* prevented the expression of FER1 in salivary glands and ovary (Hajdusek et al., 2009). Interestingly, although *Hlfer2* was silenced, we still detected HlFER2 in the midgut by western blot analysis, possibly due to its secretory nature.

Female ixodid ticks are known for their high fecundity, as they are able to lay thousands of eggs in a single batch. Failure of ferritin-silenced ticks to lay eggs may be due to insufficient nutrients, including iron, required for optimum egg production (Zhou et al., 2007). Our results suggest that HlFER2 is important during oviposition and embryonic development. This may supply iron or protect the developing oocytes and embryo from excessive iron. This was further supported by the abnormal morphology of eggs laid from *Hlfer2*-silenced females. At our laboratory, we detected host-derived transferrin in the ovary during oviposition; however, it was not detected in the eggs after laying (H. Mori, R.L.G., H.M., T.M., R.U.-S., M.M., K.F. and T.T., unpublished results), suggesting an interplay between host transferrin and the tick's HlFER2. In addition, the expression of two vitellogenin genes, *HlVg-1* and *HlVg-3*, was affected by silencing of ferritin genes. Boldbaatar et al. (Boldbaatar et al., 2010b) showed that multiple vitellogenins are essential for oocyte development and oviposition in *H. longicornis*.

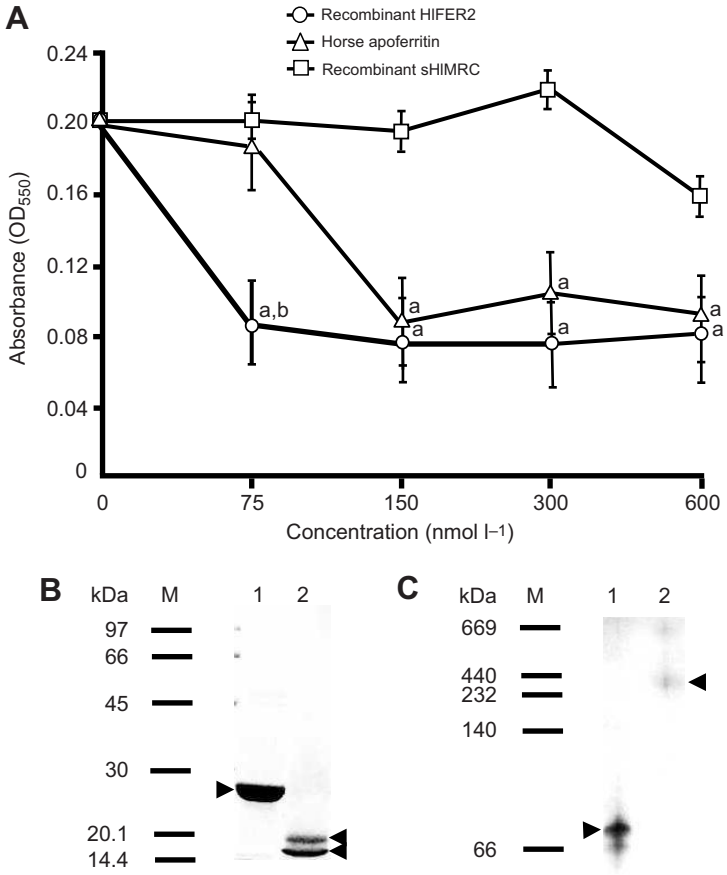

Fig. 9. Iron-binding activity and electrophoretic analyses of recombinant HlFER2. (A) Different concentrations of recombinant HlFER2 and horse apoferritin dissolved in water were incubated with $FeCl_2$. Recombinant short HlMRC was used as negative control. Ferrozine was used as an indicator agent, and absorbance at 550 nm was measured. Different lowercase letters indicate significant differences (P<0.05) *versus* negative control (a) or horse apoferritin (b). (B) SDS-PAGE (12%) and (C) native PAGE (3–10%) of recombinant HlFER2 (lane 1, 5 µg) and horse apoferritin (lane 2, 10 µg). Gels were stained with Coomassie Brilliant Blue. M, molecular weight marker. The recombinant HlFER2 and horse apoferritin bands are indicated by arrowheads.

Vitellogenin production was previously shown to be dependent on the degree of feeding (Rosell-Davis and Coons, 1989), and surpassing critical mass is important in vitellogenesis in ixodid ticks (Friesen and Kaufman, 2009). In mosquitoes, vitellogenesis is a complex event involving several hormones, triggered by the blood meal. Midgut distension from the blood meal sends a signal to the brain and starts the cascade for vitellogenesis. Amino acids from the blood meal also activate vitellogenesis (Attardo et al., 2005). The same could be true for ticks. Weiss and Kaufman (Weiss and Kaufman, 2001) showed that attainment of critical weight in the female hard tick *Amblyomma hebraeum* is necessary for production of 20-hydroxyecdysone, the hormone responsible for the initiation of vitellogenin synthesis. Moreover, they also showed that higher mass is required for ovary maturation. In both *Hlfer*-silenced groups, the body mass after a blood meal was less than half of that of the control group, and the midgut contained less blood meal. *Hlfer*-silenced ticks also have smaller ovaries after detachment from the host. Although some of the *Hlfer2*-silenced ticks laid eggs, the number was very small compared with that laid by control ticks, possibly as a result of failure of the ovary to mature. Nevertheless, the results in this study cannot fully demonstrate the direct relationship between vitellogenin and ferritin or vitellogenin and iron.

We showed that recombinant HlFER2 demonstrated apparent iron-binding activity, comparable to that of commercial horse apoferritin. During purification of recombinant ferritins from *E. coli*, we obtained insoluble recombinant HlFER1 and soluble recombinant HlFER2. All ferritins have high iron-binding capacity and can readily interact with Fe(II) ions in a solution under aerobic conditions (Harrison and Arosio, 1996). SDS-PAGE analysis showed that recombinant HlFER2 consists only of H-subunits, in contrast to horse apoferritin, which has H- and L-subunits. Interestingly, the recombinant HlFER2 did not exhibit the typical 24-mer folding of most ferritins, but still it demonstrated iron-binding activity like other recombinant ferritins (De Zoysa and Lee, 2007; Zheng et al., 2010). This may indicate a highly functional H-chain, which possesses the catalytic activity necessary for Fe(II) oxidation and iron storage.

Both HlFER1 and HlFER2 have high homology and identity with heavy-chain ferritins, and possess a conserved ferroxidase diiron center and a ferrihydrite nucleation center. Thus, both *H. longicornis* ferritins must have the same capacity for iron uptake and storage. Conversely, HlFER2, being secretory, may circulate throughout the entire tick body. Aside from iron storage and transport, HlFER2 may have additional roles. Mammals have a relatively low concentration of extracellular ferritin in the serum and synovial and cerebrospinal fluids, which has been implicated as an inflammatory indicator and possibly a component of immune response (Meyron-Holtz et al., 2011; Orino and Watanabe, 2008). In insects and other invertebrates, ferritin has been implicated in response to bacterial infections (Kremer et al., 2009; Wang et al., 2009; Zheng et al., 2010). The blood meal of ticks may also expose them to various pathogens. As vectors, several pathogens undergo multiplication in ticks before they are transmitted. It would be interesting to evaluate whether ferritin also plays a role in pathogen multiplication and transmission. Moreover, ticks are capable of long periods of starvation and may survive without feeding. Our results showed that HlFER1 and HlFER2 are both expressed in unfed midgut and ovary. We will investigate the utilization of iron during starvation periods in the ticks in future research.

In summary, we identified a secretory ferritin, HlFER2, from *H. longicornis* and characterized it with intracellular HlFER1. Our results suggest that HlFER1 is the main iron storage ferritin and the

midgut is the primary organ involved in iron metabolism. Secretory HlFER2 may play a role in iron transport and is very important in oviposition and embryonic development. RNAi experiments further demonstrated the critical importance of the iron storage function of ferritin for successful blood feeding and reproduction of the hard tick. Furthermore, knockdown of ferritins induced abnormalities in midgut cell morphology and apparently decreased their digestive activity. We also found that silencing of ferritin genes decreased the expression of vitellogenin genes, which is integral in reproduction. To our knowledge, this is the first report demonstrating the protective function of ferritin against iron in digestive cells and suggesting the correlation of the iron metabolism and vitellogenesis in hematophagous arthropods. Future studies will focus on the other functions of ferritins in the tick. Additional knowledge on the function of this iron storage protein will provide a deeper understanding of the iron metabolism in ticks that may lead to new insights for a more effective tick and pathogen control.

LIST OF ABBREVIATIONS

dsRNA	double-stranded RNA
EST	expressed sequence tag
HCH	heavy-chain homolog
HE	hematoxylin & eosin
Hlfer1	*Haemaphysalis longicornis* ferritin 1 gene
HlFER1	*H. longicornis* ferritin 1 protein
Hlfer2	*H. longicornis* ferritin 2 gene
HlFER2	*H. longicornis* ferritin 2 protein
HlVg	*H. longicornis* vitellogenin gene
IRE	iron-responsive element
IRP	iron-regulatory protein
LCH	light-chain homolog
Luc	*luciferase*
ORF	open reading frame
PBS	phosphate-buffered saline
pI	isoelectric point
RNAi	RNA interference
TEM	transmission electron microscopy
UTR	untranslated region

AUTHOR CONTRIBUTIONS

R.L.G., K.F. and T.T. were involved in the conception and design of the experiments. R.L.G., K.M.A., R.U.S., H.M., T.M., H.K. and T.T. performed the experiments. R.L.G. prepared the manuscript. All authors actively contributed to the interpretation and analysis of all results, and read and approved the final manuscript.

COMPETING INTERESTS

No competing interests declared.

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