Characterization of mitochondrial ferritin in *Drosophila*

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Mitochondrial function depends on iron-containing enzymes and proteins, whose maturation requires available iron for biosynthesis of iron–sulfur clusters and heme. Little is known about how mitochondrial iron homeostasis is maintained, although the recent discovery of a mitochondrial ferritin in mammals and plants has uncovered a potential key player in the process. Here, we show that *Drosophila melanogaster* expresses mitochondrial ferritin from an intron-containing gene. It has high similarity to the mouse and human mitochondrial ferritin sequences and, as in mammals, is expressed mainly in testis. This ferritin contains a putative mitochondrial targeting sequence and an epitope-tagged version localizes to mitochondria in transfected cells. Overexpression of mitochondrial ferritin fails to alter both total-body iron levels and iron that is bound to secretory ferritins. However, the viability of iron-deficient flies is compromised by overexpression of mitochondrial ferritin, suggesting that it may sequester iron at the expense of other important cellular functions. The conservation of mitochondrial ferritin in an insect species underscores the importance of this iron-storage molecule.

iron | metabolism | mitochondria | paraquat | testis

Mammalian cells contain two ferritin subunits, the H chain (predominant in heart) and the L chain (predominant in liver). These two subunits coassemble in different ratios into 24-subunit heteropolymers that contain a cavity in which iron is mineralized (1). Levels of ferritin H and L chains increase in iron-replete cells. Conversely, in iron-deficient cells, iron-regulatory proteins repress ferritin H and L translation by binding iron-regulatory elements in the 5′ UTRs of the respective mRNAs (2).

In humans, the gene encoding ferritin L chain is located on chromosome 19, whereas the gene for ferritin H chain is on chromosome 11, and multiple ferritin pseudogenes have been identified (3). In 2001, a DNA sequence that had been considered to be a pseudogene was shown to express a third type of ferritin that localized to mitochondria (4). The discovery was unexpected because ferritin cores had not been observed in mitochondria. Human mitochondrial ferritin shares 79% amino acid identities with ferritin H; the seven ferroxidase center residues are conserved, and the crystal structures are similar (5). The N terminus of the encoded polypeptide contains a mitochondrial targeting sequence; in mammalian mitochondria, the 30-kDa precursor is cleaved to a 22-kDa mature peptide (4), which assembles as a homopolymer. Mammalian mitochondrial ferritin mRNA does not contain an iron-regulatory element and is expressed in testes, islets of Langerhans and neurons, and at low levels in multiple other tissues (6). Elevated levels of mitochondrial ferritin have been shown to cause cytosolic iron depletion and up-regulation of the transferrin receptor in transfected cell lines (7–9). Recently, ferritin also was found in plant mitochondria (10).

A physiological function for mitochondrial ferritin has yet to be established, but abnormal accumulations of mitochondrial ferritin have been observed in some types of sideroblastic anemia (11). Also, expression of mitochondrial ferritin helps restore respiration in yeast lacking frataxin, which is a deficiency that causes mitochondrial iron overload (12).

Like mammals, insects also express ferritins composed of two types of subunits designated as heavy-chain homolog (HCH) and light-chain homolog (LCH) (see ref. 13 for review). However, in contrast to mammals, most insect ferritins are secreted proteins that are found exclusively in the secretory pathways of the cells and in the hemolymph (14). In *Drosophila melanogaster*, the genes encoding secreted ferritin subunits (Fer1HCH and Fer2LCH) are closely clustered on position 99F of the third chromosome (15), and their transcriptional regulation appears to be coordinated (16). Synthesis of Fer1HCH can be repressed by the *Drosophila* iron-regulatory proteins, but *Fer2LCH* does not have an iron-regulatory element and is not regulated by iron-regulatory proteins. Also, an alternatively spliced variant of *Fer1HCH* lacking the iron-regulatory element is preferentially expressed in the presence of high iron levels (17). Recent characterization of the crystal structure of a native ferritin secreted from lepidopteran insect cells revealed a heteropolymer composed of 12 HCH and 12 LCH subunits, arranged with a tetrahedral symmetry (18). This arrangement is in sharp contrast to vertebrate ferritin structures, which have octahedral symmetry, and it is not yet clear whether tetrahedral symmetry is a general property of all insect ferritins. However, the above-mentioned differences between mammalian and insect ferritins (and transferrins; see ref. 13) indicate that insect cells may employ mechanisms to maintain iron homeostasis that vary from the mechanisms that are known to operate in mammals.

Annotation of the sequenced genome of *D. melanogaster* has revealed a third ferritin gene (*CG4349*) that is located on the X chromosome. It was suggested initially that this gene could encode a ferritin subunit belonging to a cytosolic ferritin because cytosolic ferritins have also been observed in insects (19) and ticks (20). Here, we establish that *CG4349* (*Fer3HCH*) encodes a mitochondrial ferritin subunit. In a striking similarity to mammalian mitochondrial ferritin, *Drosophila Fer3HCH* is expressed most highly in testis, but unlike the intronless mammalian mitochondrial ferritin gene, the *Fer3HCH* transcript undergoes splicing. We have generated flies that overexpress Fer3HCH under control of the Gal4–upstream activating sequence (UAS) system (21), and we report the initial characterization of this transgenic model.

Results

Genomic Organization of *CG4349* and Cloning of a cDNA. In addition to *Fer1HCH* and *Fer2LCH*, the *D. melanogaster* genome also

Conflict of interest statement: No conflicts declared.

Abbreviations: BPS, bathophenanthroline disulfonic acid; HCH, heavy-chain homolog; LCH, light-chain homolog; TrxR-1, thioredoxin reductase 1; UAS, upstream activating sequence.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ373966 (genomic *Fer3HCH*) and DQ373967 (*Fer3HCH* cDNA)].

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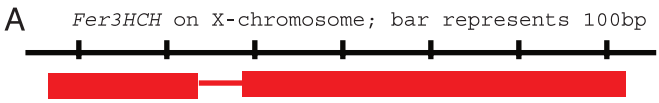

B

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droso MAWCFRDIRR HMC.......  ..........  ..........
human MLSCFRLLSR HISPSLASLR PVRCCFALPL  RWAPGRPLDP
mouse MLSCFWFFSK HISSALMSLP RVLHRFTAPQ  CLASRYPLGP

droso ..........  ..........  .....MLVRQ  NFAKSCEKKL
human RQIAPRRPLA  AAASSRDPTG  PAAGPSRVRQ  NFHPDSEAAI
mouse LLASPRRLLA  SVASSQDSTR  ....PSRVRQ  NFHPDSEAAI

droso NDQINMELKA  SHQYLAMAYH  FDRSDISSPG  MHRFFLKASV
human NRQINLELYA  SYVYLSMAYY  FSRDDVALNN  FSRYFLHQSR
mouse NRQINLELYA  SYVYLSMAYY  FSRDDVALYN  FSKYFLRQSL

droso EEREHAEKIM  TYMNKRGGLI  ILSSVPQP.L  PCFASTLDAL
human EETEHAEKLM  RLQNQRGGRI  RLQDIKKPEQ  DDWESGLHAM
mouse EEREHAEKLM  KLQNQRGGRI  CLQDIKKPDK  DDWECGLRAM

droso KHAMKMELEV  NKHLLDLHAL  AGKEADPNLC  DFIEANFLQE
human ECALLLEKNV  NQSLLELHAL  ASDKGDPHLC  DFLETYYLNE
mouse ECALLLGKNV  NQSLLDLHTL  ASEKGDPHLC  DFLETHYLHE

droso QVDGQKILAD  YISQLEKAQN  ...QVGEFLF  DKYMGSGMHPAK
human QVKSIKELGD  HVHNLVKMGA  PDAGLAEYLF  DTHTLGNENKQN
mouse QVKSIKELGD  HVHNLVTMGA  PAAGLAEYLF  DKHTLGSESKH
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Fig. 1. Genomic organization and protein alignment of Fer3HCH. (*A*) *CG4349* (*Fer3HCH*) lies on the X chromosome and contains one intron. (*B*) Alignment of Fer3HCH to human and mouse mitochondrial ferritin sequences. Conserved amino acid residues important for ferroxidase activity are shown in red.

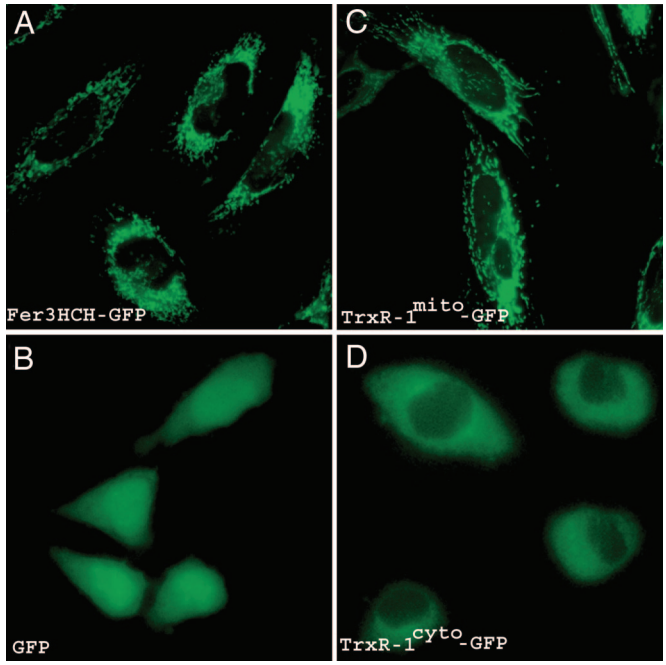

Fig. 2. *D. melanogaster* expresses a mitochondrial ferritin. HeLa cells were transfected with indicated plasmids expressing GFP fusion proteins. The transfected cells were visualized by using wide-field fluorescence microscopy. (*A* and *B*) Fer3HCH–GFP (*A*) is expressed in the mitochondria, in contrast to free GFP (*B*), which localizes to the cytoplasm and the nucleus. (*C* and *D*) The pattern of Fer3HCH–GFP is identical to TrxR-1^{mito}–GFP (*C*) and clearly distinct from TrxR-1^{cyto}–GFP (*D*).

contains a third ferritin homolog, *CG4349* (22). *CG4349* is located on the X chromosome at polytene region 11D11. Because no expressed sequence tags were available, we cloned cDNAs from early pupae and adult flies by RT-PCR. PCR primers were designed to amplify a fragment spanning the entire coding region plus 3 bp of the 5′ UTR and 66 bp of the 3′ UTR. Products of two lengths were obtained from each reaction, representing amplification products of cDNA and genomic DNA. Sequencing of the cloned products confirmed the presence of a single intron of 66 bp, which is 4 bp longer than the intron that was predicted in the genome annotation of *CG4349* (Fig. 1*A*).

***CG4349* Encodes a Ferritin HCH Subunit That Is Similar to Human Mitochondrial Ferritin.** A multiple amino acid sequence alignment of the *Drosophila* ferritin subunit with mammalian mitochondrial ferritin subunits is shown in Fig. 1*B*. The *CG4349* gene encodes a polypeptide of 186 aa. Importantly, all seven amino acid residues that form the ferroxidase center necessary for oxidation and sequestration of iron (red in Fig. 1*B*) are conserved in the deduced polypeptide. Thus, *CG4349* clearly encodes a HCH ferritin subunit, which we named *Fer3HCH*, consistent with the nomenclature used for *Drosophila* ferritins (15). Partial similarity of the N-terminal sequence of the predicted Fer3HCH protein with the long mitochondrial-targeting sequence of human and mouse mitochondrial ferritins suggested that Fer3HCH could be targeted also to the mitochondria.

Fer3HCH Is Targeted to the Mitochondria. To experimentally determine where Fer3HCH localizes in the cell, we used a human cell culture system that has been used successfully in showing that *Drosophila thioredoxin reductase 1* (*TrxR-1*) encodes distinct mitochondrial and cytosolic isoforms (23). We fused GFP to the C terminus of Fer3HCH to allow normal processing of the putative mitochondrial leader sequence, and we observed transfected HeLa cells by fluorescence microscopy. The results clearly indicated that the Fer3HCH–GFP fusion protein accumulates in the mitochondria in a pattern that is identical to that of TrxR-1^{mito} and distinct from that of TrxR-1^{cyto} and free GFP (Fig. 2*C*). This result strongly suggests that Fer3HCH represents the *Drosophila* homolog of mammalian mitochondrial ferritin.

Fer3HCH Is Preferentially Expressed in Testis. In both humans and mice, mitochondrial ferritin is highly expressed in testis and expressed at low levels in other tissues. To determine the expression pattern of *Fer3HCH* in *Drosophila*, we assessed the relative amount of *Fer3HCH* mRNA that was present in different tissues and at different stages of development, including iron-fed flies in all experiments to test whether iron induces the expression of *Fer3HCH*.

Northern blot analysis of different developmental stages of flies revealed undetectable levels of *Fer3HCH* message during development and only a faint signal appearing at pupal stages and in a sample from adult flies of mixed sex, not influenced by dietary iron treatment (Fig. 3*A*). Next, we analyzed mRNA levels of *Fer3HCH* in the three major body segments (abdomen, thorax, and head) of male and female *Drosophila* adults that were raised on both normal and iron-supplemented diets (Fig. 3*B*). *Fer3HCH* expression was detected only in male abdomens, indicating that it is most likely to be expressed in male gonads. The amount of iron that was present in food had no effect on this expression. This result has been confirmed by microarray gene-expression comparisons that were performed on adult flies with or without dietary iron supplementation (F.M., V. Gupta, and T.A.R., unpublished data).

To confirm that *Fer3HCH* expression was testis-specific, we also analyzed multiple microarray gene-expression experiments

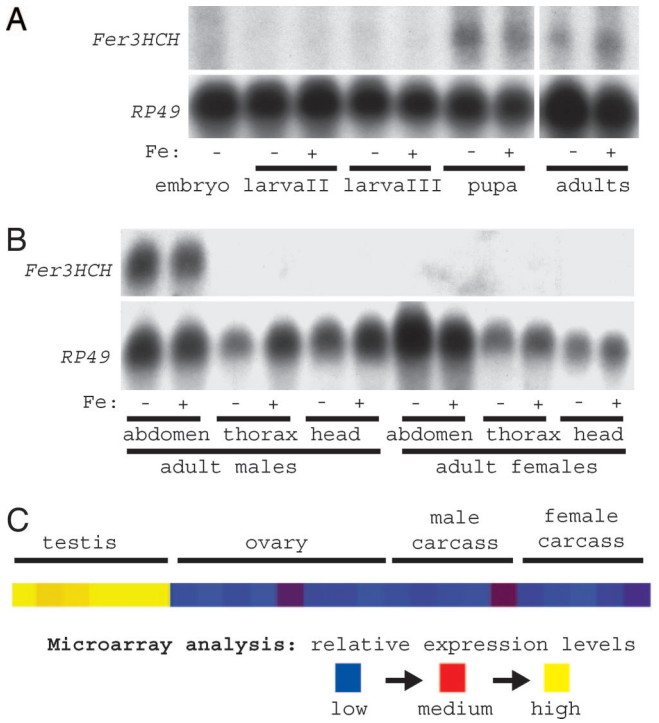

Fig. 3. *Fer3HCH* is highly expressed in testis. (*A*) Developmental Northern blot analysis hybridized consecutively with a *Fer3HCH*-specific probe and with Rp49 for loading control. Addition of 5 mM $FeCl_3$ to food at the different developmental stages is shown. Note that expression appears only at the pupal and adult stages and is unaffected by iron treatment. (*B*) RNA from the different body segments of sexed adults was analyzed by Northern blotting. Expression was detected only in male abdomens. (*C*) Expression levels of *Fer3HCH* in 24 different microarray experiments normalized to each other (24). Each box represents one sample from experiments denoted by line above the relative expression data. High levels of expression were detected only in testis.

comparing global gene-expression levels in testis, ovary, and soma of both sexes (24). Normalized, color-coded expression data for *Fer3HCH* from these experiments are shown in Fig. 3*C*, and, consistent with the Northern blot analysis described above, high expression levels were detected only in testis.

Fer3HCH Overexpression *in Vivo* Does Not Affect Levels of Fer1HCH or Fer2LCH. To study the effects of mitochondrial ferritin overexpression *in vivo*, transgenic flies that contain the *Fer3HCH* cDNA under the control of the yeast UAS cis-acting element were generated by *P* element-mediated transformation (25). Crossing of these flies to different available Gal4 drivers resulted in tissue-specific expression of *Fer3HCH* (21). Two different *UAS-Fer3HCH* transgene insertions were isolated and both mapped on the third chromosome. Also, we used recombination to generate a chromosome that contains both transgene insertions, which allows dosage-dependent overexpression of *Fer3HCH* (Fig. 4*A*). To achieve overexpression, we crossed the transgenic lines to the *Actin-Gal4* (ubiquitously expressing) driver line. As a control, we crossed a strain lacking the transgenes of the same genetic background. Female flies were homogenized, and lysates were analyzed by Western blotting probed with serum from rabbits that were immunized against Fer3HCH peptides. Migrating between the 22- and 16-kDa marker proteins, a band appears with increasing intensity in flies that overexpress one or two copies of the transgene but is absent from control flies (Fig. 4*A*). Thus, consistent with the gene-expression data, mitochondrial ferritin was not found to be an abundant protein in WT animals but could be overexpressed *in vivo*.

Transfection of human cells with human mitochondrial ferritin resulted in cytosolic iron depletion, cytosolic ferritin inhibition, and up-regulation of the transferrin receptor (7–9). Because *Drosophila* has no recognizable transferrin receptor, we could not use transferrin receptor expression as an indicator of iron status, and instead, we analyzed the levels of Fer1HCH and Fer2LCH in flies that overexpress Fer3HCH. For this analysis, we generated and affinity-purified rabbit polyclonal Abs to peptides of Fer1HCH and Fer2LCH, and we confirm that levels of Fer1HCH and Fer2LCH vary, as expected according to iron status in *Drosophila* adults (Fig. 5*A*). Western blot analysis indicated that levels of Fer1HCH and Fer2LCH did not change in flies overexpressing Fer3HCH as compared with the control (Fig. 4*A*). The direct implication of this result is that cellular iron status was not altered significantly by Fer3HCH overexpression, because such changes were predicted to affect Fer1HCH and Fer2LCH (see Fig. 5*A*).

To further eliminate putative genetic background influences on Fer1HCH and Fer2LCH expression or gender-specific differences, we also used male adult flies and added to our analysis a strain that contained both *UAS-Fer3HCH* transgenes but lacked the *Actin-Gal4* driver, as well as a strain that lacked both *UAS-Fer3HCH* and *Actin-Gal4*. Levels of Fer1HCH and Fer2LCH remained constant in all four genotypes (three controls and *Actin*-driven Fer3HCH overexpression), indicating that levels of Fer1HCH and Fer2LCH do not change in response to overexpression of Fer3HCH (Fig. 4*B*).

It has been suggested that ferritin is made in insect fat bodies and secreted to the hemolymph (26, 27), and therefore, we analyzed Fer1HCH and Fer2LCH levels when Fer3HCH was expressed under the control of a fat-body Gal4 driver. For both sexes, levels of Fer1HCH and Fer2LCH were unaffected by fat-body Fer3HCH overexpression (data not shown).

Last, to demonstrate that overexpressed Fer3HCH assembles in holopolymers, we analyzed the fly extracts under nonreducing conditions by SDS/PAGE (Fig. 4*C*). Fer3HCH homopolymers were detected as high-molecular-mass complexes. Fer1HCH–Fer2LCH heteropolymers migrated at an apparent molecular mass of 150-kDa with the addition of SDS, possibly because of a dissociation of the 24-mer into 6-mer, which could remain assembled through intersubunit disulfide bonds (18). Samples that were derived from flies that were fed with radioactive Fe^{55} were analyzed by autoradiography, but iron could be detected only in association with the Fer1HCH–Fer2LCH heteropolymer, suggesting that Fer3HCH homopolymers are relatively iron-poor when overexpressed in WT flies. This observation is consistent with the unaltered levels of heteropolymeric ferritin in Fer3HCH-overexpressing flies.

Overexpression of Fer3HCH Does Not Affect Total Iron Levels in the Whole Organism. To corroborate that Fer3HCH is not heavily iron loaded, we also sought to determine whether failure of Fer3HCH overexpression to induce relative cytoplasmic iron deficiency (and, thus, to decrease Fer1HCH and Fer2LCH expression) could be explained instead by increased dietary iron absorption. We measured total-body iron by a modification of the ferrozine assay, which normally reacts only to ferrous iron. Whole-fly lysates were boiled in acid (to release iron from proteins, including ferritin); iron was reduced by addition of ascorbate; samples were incubated with ferrozine; and iron levels were monitored by colorimetric measurements (see *Materials and Methods*). First, we validated the assay by testing flies that were placed on iron diets (Fig. 5*B*). Supplementation of food with 200 μM iron chelator bathophenanthroline disulfonic acid (BPS) dropped body iron stores to half of normal. Supplementation of food with 5 mM ferric ammonium citrate (FAC)

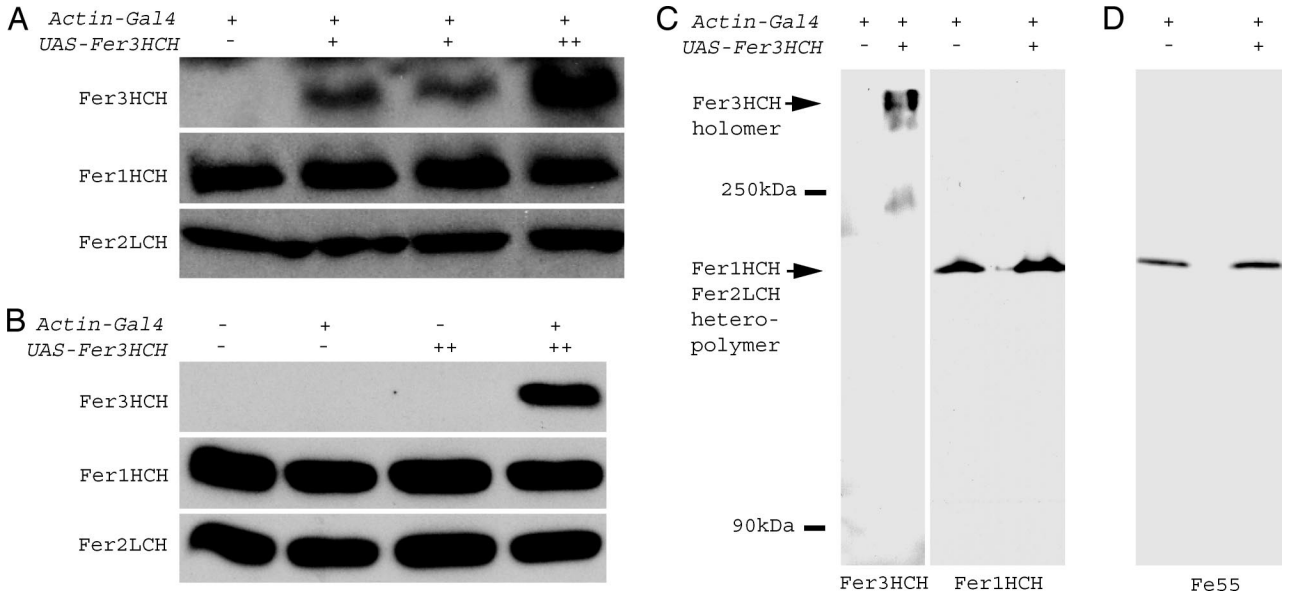

Fig. 4. Overexpression of Fer3HCH homopolymers *in vivo* does not affect levels of Fer1HCH or Fer2LCH. (*A*) Western blot analyses of female whole-fly lysates separated under reducing conditions by SDS/PAGE and probed with antisera to Fer3HCH, Fer1HCH, and Fer2LCH. Fer3HCH overexpression was achieved only in the presence of both *Actin-Gal4* and *UAS-Fer3HCH* (indicated by +). The sensitivity of our antisera allows for detection of Fer3HCH only in flies that overexpress the protein and the signal increases in flies with two copies of the transgene (indicated by ++). (*B*) Western blot analyses of male whole-fly lysates including additional controls showing that levels of Fer1HCH and Fer2LCH are unaffected by Fer3HCH overexpression. (*C*) Samples separated under nonreducing conditions by SDS/PAGE. Note the higher-molecular-mass species, which is detected by the Fer3HCH antisera only in samples from overexpressing flies. (*D*) Autoradiography on samples from flies that fed on Fe^{55} for 48 h before they were killed. Only the fragmented Fer1HCH–Fer2LCH heteropolymer accumulates detectable levels of Fe^{55}.

resulted in a 4-fold increase of total iron levels. In sharp contrast, total iron levels were unaffected by overexpression of Fer3HCH either ubiquitously or in fat bodies (Fig. 5*C*).

Overexpression of Fer3HCH Does Not Affect Development. Adult flies overexpressing *Fer3HCH* under the control of the *Actin-Gal4* driver showed no apparent phenotypes. To examine possible effects at earlier life stages, we set up a cross, which by Mendelian genetics was predicted to result in equal ratios of progeny overexpressing Fer3HCH to controls. Altered sibling ratios might suggest a physiological role for Fer3HCH in development, either under normal conditions or high iron concentrations in the food or in the presence of BPS. We scored the genotype of every fly that eclosed from these crosses and calculated χ^2 values within each sex for our final ratios of overexpression vs. control flies raised in the different diets (Fig. 6*A*). Each cross fell below the threshold for $P = 0.05$; therefore, the sibling ratios for overexpression vs. control flies do not deviate from 1:1 in any of the tested diets.

Overexpression of Fer3HCH Impacts Female Life Span Under Iron-Limiting Conditions. In search of a discernible phenotype related to *Fer3HCH* overexpression, we monitored the life span of female and male flies, raised under different iron diets. Under adequate dietary iron, there was no effect of *Fer3HCH* overexpression on life span. In contrast, female flies that developed and were maintained throughout their life span on food containing 0.2 mM BPS showed a 25% decrease in their mean life span (Fig. 6*B*). This phenotype was negligible in male flies (data not shown).

Overexpression of Fer3HCH Protects Females from Paraquat Toxicity. We also noted that Fer3HCH confers a robust protection from paraquat toxicity in young adult female flies (Fig. 6*C*). Our initial hypothesis was that protection from paraquat toxicity was attributable to a direct effect of iron sequestration in mitochondrial ferritin and inhibition of iron-catalyzed free radical generation. However, the protection is again gender-specific, making such a hypothesis unattractive. We believe that a minor redistribution of iron in the overexpressed Fer3HCH results in a conspicuous reprogramming of the female's developmental program, affecting oogenesis, resistance to stress, and life span. Similar phenotypes have been reproduced with Fer1HCH and Fer2LCH cooverexpression (F.M. and T.A.R., unpublished data). Together, we think our data offer experimental evidence that Fer3HCH can affect iron homeostasis in flies, although to a much lesser extent than predicted, based on experiments in human cell culture systems (7–9).

Discussion

Mitochondrial ferritin was first characterized in mammals, and it was recently shown that plants also have ferritin localized in the mitochondria. Here, we describe a similar ferritin (Fer3HCH) in *D. melanogaster*. With the addition of insects, mitochondrial ferritin has now been described in another phylum within the animal kingdom, strengthening the idea that it has an important role in iron metabolism. We show that the Fer3HCH sequence is similar to both mouse and human mitochondrial ferritin, although it has a shorter leader sequence that is nevertheless sufficient to target the protein to mitochondria.

In striking similarity to the expression pattern of human and mouse mitochondrial ferritin, *Fer3HCH* is highly expressed in the *D. melanogaster* testis. It was suggested that mitochondrial ferritin is highly expressed in mammalian testis because the spermatids and Leydig cells of the testis have a high concentration of mitochondria (6). Leydig cells contain unusual mitochondria that have specialized and morphologically distinctive cristae that are involved in conversion of cholesterol to pregnenolone, a precursor of testosterone (28). Mitochondrial conversion of cholesterol requires several heme-containing proteins and ferredoxin, an iron–sulfur protein (29). Because expression levels of mitochondrial ferritin are low in other mitochondria-rich tissues

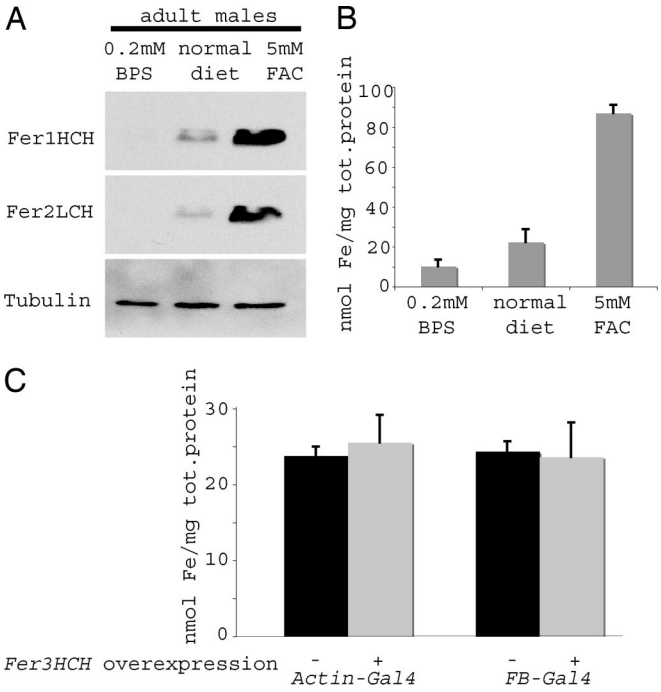

Fig. 5. Overexpression of Fer3HCH *in vivo* does not affect total-body iron stores. (*A*) Western blot analyses of adult males that were raised throughout their development on food containing BPS (0.2 mM), no additives, or ferric ammonium citrate (FAC) (5 mM). Endogenous Fer3HCH was below detection limits (data not shown); tubulin was used as loading control. (*B*) Measurement of total iron in lysates from the same flies as in *A* indicates that manipulation of iron in the diets changes total-body iron levels. (*C*) Measurement of total iron in flies overexpressing Fer3HCH ubiquitously (*Actin* driver +) or in the fat bodies (*FB* driver +) indicates that Fer3HCH overexpression did not increase total-body iron content.

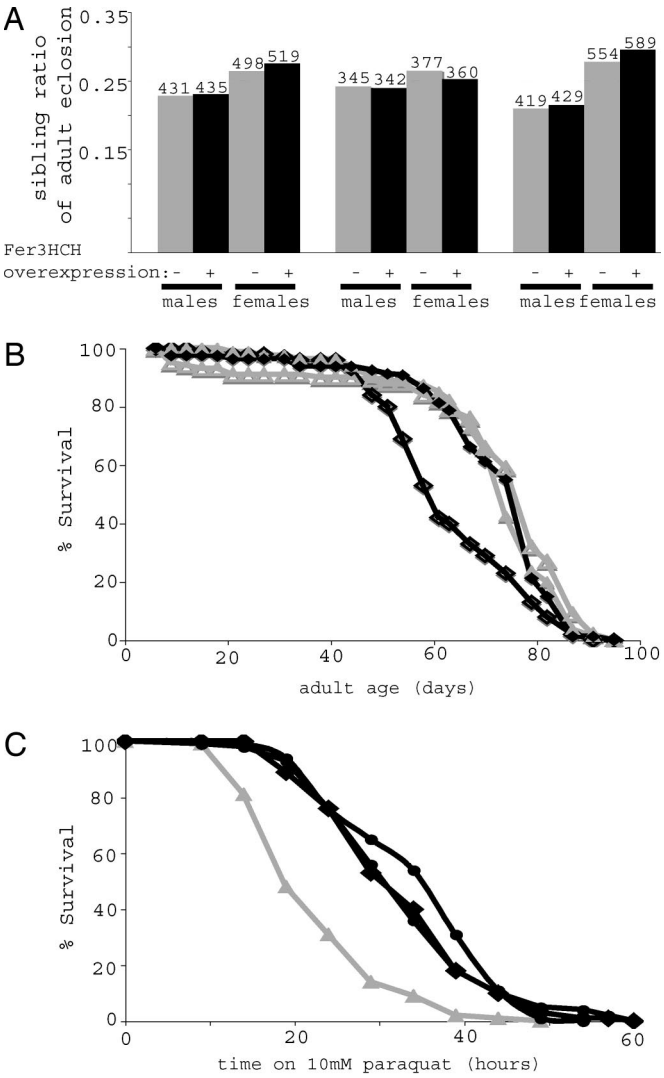

Fig. 6. Increased resistance to paraquat toxicity but decreased life span under iron-limiting conditions in female flies overexpressing *Fer3HCH*. (*A*) Overexpression of Fer3HCH does not influence adult eclosion rates, independent of dietary iron levels. Bars represent the ratios of total progeny from the cross *Actin-Gal4/Cyo* X *UAS-Fer3HCH/UAS-Fer3HCH* scored by gender and by the presence or absence of the *Actin-Gal4* driver. Numbers above the bars give the number of actual flies that were counted. (*B*) Life span of female *Actin-Gal4/+; +/+* (triangles) and *Actin-Gal4/+; UAS-Fer3HCH/+* flies (rhomboids) raised on normal food (filled triangles and rhomboids) or on food supplemented with 0.2 mM BPS (open triangles and rhomboids). Note that overexpression of Fer3HCH impacts female life span under iron-limiting conditions. (*C*) Survival on 10 mM paraquat is enhanced in female flies overexpressing Fer3HCH. Genotypes are *Actin-Gal4/+; +/+* (triangles), *Actin-Gal4/+; UAS-Fer3HCH/+* flies (circles, both independent transgenes) and *Actin-Gal4/+; UAS-Fer3HCH,UAS-Fer3HCH/+* flies (rhomboids).

such as muscle and brain, an additional explanation for high testis expression may be related to the unusual morphology and steroidogenic role of Leydig cell mitochondria. The insect testis is structurally and functionally different from that of mammals and is not known to produce ecdysteroids. The high expression level of Fer3HCH in *Drosophila* testis compared with the low expression levels in other mitochondria-rich tissues (such as the thoracic muscles) suggests a possible link to the dramatic transformations of mitochondria during the spermatid development (30). Most important, testes in both mammals and insects are separated from the systemic circulation and, therefore, may need special mechanisms for sequestration of excess mitochondrial iron. Expression of a specialized mitochondrial ferritin may reflect such a mechanism.

Whereas previous studies examined the effects of mitochondrial ferritin overexpression *in vitro*, we created a *Drosophila* model to study the *in vivo* effects of Fer3HCH overexpression. In contrast to the earlier results in mammalian cells, we detected no change in levels of the secreted ferritins Fer1HCH or Fer2LCH in adult flies upon overexpression of Fer3HCH. In our experimental system, Fer3HCH homopolymers remained iron-poor, possibly reflecting the function of other homeostatic mechanisms for iron in the mitochondria, which render iron unavailable to ferritin. However, because we analyzed whole-fly lysates, subtle changes in iron loading of Fer3HCH or in levels of heteropolymeric ferritin retained in the secretory pathway of certain cell types (such as in the iron region of the midgut, where intracellular ferritin is most abundant) (14) may have gone unnoticed.

We also investigated whether development is affected in our Fer3HCH overexpressing flies under limiting, normal, or excessive iron conditions. Our results suggest that overexpression of Fer3HCH does not affect developmental timing or survival to adulthood. This finding is in contrast to flies simultaneously overexpressing Fer1HCH and Fer2LCH under control of the *Actin* driver, which show a developmental disadvantage that becomes marked as iron levels in the diet decrease (F.M. and T.A.R., unpublished data). However, female-specific protection from paraquat and life-span decrease under limiting iron conditions are phenotypes that are also detected when the secreted ferritins are overexpressed. Collectively, these results suggest

that mitochondrial ferritin overexpression may have subtle effects on global iron metabolism. Given the recent development of a *Drosophila* model for suppression of frataxin expression it would be interesting to test whether Fer3HCH can function protectively under pathological conditions (31). Because mitochondrial ferritin is a nuclear-encoded protein and its levels can change dramatically (as shown in sideroblastic anemia patients), it is likely that a complex nuclear–mitochondrial communication system exists within the cell to protect mitochondria from iron overload (32). Our *Drosophila* model of mitochondrial ferritin overexpression offers a valuable tool for manipulating mitochondrial iron metabolism.

Materials and Methods

***Drosophila* Stocks and Generation of Fer3HCH Overexpressors.** All *D. melanogaster* strains were maintained on standard cornmeal/yeast/agar medium at 25°C, and all iron manipulations are as described. For Northern blot analysis, Ore-R WT flies were used. To create *UAS-Fer3HCH* the corresponding cDNA was cloned in the PUAST vector and sent for injection into embryos at Genetic Services, Inc. (Sudbury, MA). *Actin-Gal4* and *fat body-Gal4* strains were as described in ref. 33.

Cell Culture. To create the Fer3HCH·GFP plasmid, the Fer3HCH cDNA was PCR amplified, gel-purified, and cloned into the pEGFP-N1 vector (Clontech). HeLa cells were maintained at 37°C in standard cell culture medium, transfected by using the Lipofectamine 2000 reagent, and visualized with an Eclipse E600 fluorescence microscope (Nikon).

Abs and Probes. Protein and RNA isolation as well as Western and Northern blot analyses were performed as described in ref. 16. The Fer3HCH and Rp49 genes were used for synthesis of the respective probes. Synthetic multiple antigenic peptides (MAPs) corresponding to amino acids 106–121 of Fer1HCH, 150–165 of Fer2LCH, and 25–40 of Fer3HCH were synthesized by Biosynthesis (Lewisville, TX) and injected into rabbits for generation of polyclonal Abs (Covance Laboratories, Vienna, VA). Anti-Fer1HCH and anti-Fer2LCH were affinity purified on columns made with the corresponding MAP. Anti-Fer3HCH was not purified and was used as serum. Anti-tubulin mouse mAb ascites fluid (clone DM1A) was obtained from Sigma. We used the following Ab concentrations: Fer1HCH, 1:2,000; Fer2LCH, 1:1,250; Fer3HCH, 1:750; and tubulin, 1:4,300.

Microarray Analysis. Log2-normalized intensities from all experiments were zero centered (Gene Expression Omnibus platform GPL-20) to create an adjusted intensity for the heat diagram, as shown by different colors (24).

Iron Extraction and Ferrozine Assay. We homogenized 10 females or 12 males in 125 μl of lysis buffer. They then were centrifuged twice at 16,000 $\times$ *g* in a bench-top centrifuge, and 80 μl was recovered. Protein concentration was determined by using the Bradford assay. We added 17 μl of concentrated HCl to 77 μl of sample (or lysis buffer, for control). It then was heated for 20 min at 95°C and centrifuged at 16,000 $\times$ *g* for 2 min. We placed 66 μl of supernatant in a fresh tube and recentrifuged at 16,000 $\times$ *g* for 2 min. We added 50 μl of supernatant to 20 μl of 75 mM ascorbate, and it then was vortexed and spun down. Next, 20 μl of 10 mM ferrozine was added, vortexed, and spun down. Last, 40 μl of saturated ammonium acetate was added to each tube and vortexed, and absorbance was measured at 562 nm. The following formula was used to calculate concentration of iron: $[\mathrm{Fe}]\ (\mathrm{pmol}/\mu\mathrm{l}) = [(\mathrm{DOD}_{562} \times 94/77 \times 130/50)/27{,}900] \times 10^6$.

Paraquat Resistance and Life-Span Determinations. For paraquat and life-span experiments, the progeny of young parental crosses monitored for constant larval densities were collected after a 24-h eclosion window. Males and females were kept together for 3 additional days to allow for mating, and they were then separated and used in the two assays, which were performed as described in ref. 33.

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