Insertion mutants in *Drosophila melanogaster* Hsc20 halt larval growth and lead to reduced iron–sulfur cluster enzyme activities and impaired iron homeostasis

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**Abstract** Despite the prominence of iron–sulfur cluster (ISC) proteins in bioenergetics, intermediary metabolism, and redox regulation of cellular, mitochondrial, and nuclear processes, these proteins have been given scarce attention in *Drosophila*. Moreover, biosynthesis and delivery of ISCs to target proteins requires a highly regulated molecular network that spans different cellular compartments. The only *Drosophila* ISC biosynthetic protein studied to date is frataxin, in attempts to model Friedreich’s ataxia, a disease arising from reduced expression of the human frataxin homologue. One of several proteins involved in ISC biogenesis is heat shock protein cognate 20 (Hsc20). Here we characterize two piggyBac insertion mutants in *Drosophila* Hsc20 that display larval growth arrest and deficiencies in aconitase and succinate dehydrogenase activities, but not in isocitrate dehydrogenase activity; phenotypes also observed with ubiquitous frataxin RNA interference. Furthermore, a disruption of iron homeostasis in the mutant flies was evidenced by an apparent reduction in induction of intestinal ferritin with ferric iron accumulating in a subcellular pattern reminiscent of mitochondria. These phenotypes were specific to intestinal cell types that regulate ferritin expression, but were notably absent in the iron cells where ferritin is constitutively expressed and apparently translated independently of iron regulatory protein 1A. Hsc20 mutant flies represent an independent tool to disrupt ISC biogenesis in vivo without using the RNA interference machinery.

**Keywords** Mitochondria · Iron–sulfur clusters · DnaJ protein · Iron regulatory protein · Iron regulatory element

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| Fer1HCH      | Ferritin-1 heavy chain homologue |
| GFP          | Green fluorescent protein |
| Hsc20        | Heat shock protein cognate 20 |
| ISC          | Iron–sulfur cluster |
| modENCODE    | Model Organism Encyclopedia of DNA Elements |
| PBS          | Phosphate-buffered saline |
| RNAi         | RNA interference |
| Tris         | Tris(hydroxymethyl)aminomethane |
| UAS          | Upstream activating sequence |

**Introduction**

Iron–sulfur clusters (ISCs) may have participated in reactions leading to the origin of life on Earth [1, 2]. They are central to most key processes sustaining living ecosystems, including carbon and nitrogen fixation [3]. In animals, ISC proteins are involved in oxidative phosphorylation and the regulation of iron homeostasis [4, 5]. ISC proteins are also conspicuously abundant in the nucleus, but the specific
functions for the ISCs in DNA replication and repair are currently under investigation [6–9]. Cells actively build and deliver ISCs to target proteins [10–13]; defects in this process lead to disease, notably Friedreich’s ataxia [14–16], which is caused by the expansion of a GAA trinucleotide repeat element in an intron of the human frataxin gene [17]. The study of related human disorders has revealed tissue-specific requirements of specific ISC biosynthesis genes [18–20].

One factor considered important for delivery of ISCs to target proteins, in particular under conditions of oxidative stress, is heat shock protein cognate 20 (Hsc20) [21, 22]. The yeast homologue of Hsc20 is known as Jac1 and was recovered from a genetic screen as a suppressor of superoxide dismutase deficiency [23]. Reduced activity of Jac1 resulted in a decrease in activity of iron/sulfur-containing mitochondrial proteins and an accumulation of iron in mitochondria [24–26]. Jac1 interacts with the iron–sulfur scaffold protein Isu1p [27] and this interaction appears to be conserved in evolution [21, 28]. Hsc20 proteins from higher animals contain a metal-binding, cysteine-rich N-terminal domain, which is important for the integrity and function of the human co-chaperone [21, 29].

ISC biosynthesis has been studied in Drosophila, following the cloning of dfh, the fly homologue of the human frataxin gene [30]. A number of laboratories have used RNA interference (RNAi) induced by the heterologous yeast Gal4/upstream activating sequence (UAS) transgenic induction system [31] to model Friedreich’s ataxia in Drosophila [32–36]. Strong ubiquitous reduction in Hsc20 resulted in giant long-lived larvae that failed to initiate metamorphosis and had defects in ISC enzymes [32], whereas more moderate reductions in expression resulted in adult flies with locomotion defects and increased sensitivity to oxidative stress [33]. Scavenging of hydrogen peroxide but not of superoxide rescued many dfh phenotypes [34], including the reversal of inactivation of aconitase, whose ISC is a bona fide target for superoxide [37]. This result is consistent with a signaling role for hydrogen peroxide as recently uncovered in the fly hematopoietic and wound-healing processes [38–40]. Reduced dfh expression in the central nervous system led to defective mitochondrial axonal transport and membrane potential when RNAi was induced in neurons [35], and accumulation of lipids and lipid peroxidation when RNAi was induced in glia [36]. These studies and also observations that ubiquitous overexpression of dfh affected specifically the development of embryonic muscles [41] have highlighted the variable tissue-specific roles also for Drosophila dfh. As no animal models are available beyond dfh RNAi flies and frataxin knockout mice [42], we undertook to characterize two transgenic insertions disrupting the Drosophila Hsc20 homologue.

**Materials and methods**

**Drosophila stocks**

All Drosophila melanogaster strains were maintained on standard cornmeal/yeast/agar medium at 25 °C. Iron supplementation was in the form of ferric ammonium citrate. PBac[PB]i(3)72Do^{coso18}$/TM6 and PBac[WH]i(3)72-Do^{02457}$/TM6, Tb were from the Exelixis collection at Harvard Medical School. Both insertions were rebalanced to the TM3, P[Gal4-Kr.C]/DC2, P[UAS-GFP.S65T]/DC10, Sh^® fluorescent balancer ( GFP is green fluorescent protein) from Bloomington stock no. 5195 to identify the time of larval lethality and select homozygous mutant larvae for further analysis. The resulting stocks are abbreviated Hsc20^{5018}/TM3, Kr-GFP, Sb and Hsc20^{2457}/TM3, Kr-GFP, Sh, respectively, in the text. Actin-Gal4 used for rescue experiments and Fer1HCH_{G188} (Fer1HCH is ferritin-1 heavy chain homologue) used for visualization of intestinal ferritin have been described elsewhere [43].

**RNA isolation and reverse transcription PCR**

Total RNA was isolated from larvae using a modified Trizol method [44] and was purified using an RNasy Mini kit (QIAGEN). The RNase-free DNase set (QIAGEN) was applied for on-column digestion of residual DNA. Total RNA of each sample was first reverse-transcribed into complementary DNA using the SuperScript III system (Invitrogen). The sequence encoding the full-length Hsc20 protein was amplified by PCR using the total match primers

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5'-ATGAGTGATGAATTATCAATGCTTAAAA GTGCCT3'-5'
\]

and

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5'-TCAGCTGCCCAGCAAACTTTGTTG 18
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Eagles medium with 4.5 g/l glucose, supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C and 5% CO2. Cells were seeded in microwell Lab-Tek chambered cover glass (Nunc International), and Fugene 6 (Roche) was used for transient transfections with mCherry-tagged Hsc20 according to the recommendations of the manufacturers. Immunofluorescence was imaged with a confocal microscope system (LSM 510 META; Zeiss) typically 24–36 h after transfection. For imaging red fluorescence, the 543-nm line of an He–Ne laser was used with a 488 nm/543 nm dichroic mirror, and the fluorescence was collected with a 560-nm long-pass filter.

Enzyme assays

Three third instar larvae were transferred to a 1.5-ml microcentrifuge tube and were washed with 1× phosphate-buffered saline (PBS). Subsequently they were homogenized in about 5 vol of ice-cold lysis buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl at pH 8.0, containing 1% (v/v) NP-40 (Fluka), and EDTA-free protease inhibitor (one tablet per 10 ml) (Roche)] with a disposable plastic pestle and briefly sonicated on ice. The homogenates were cleared by centrifugation (16,000g, 4 °C, 15 min). Total aconitase activity was determined by following the reduction of NADP+ at 340 nm in the subsequent isocitrate dehydrogenase reaction. Five microliters of the homogenate was added to 50 μl buffer R1 [50 mM Tris/HCl, pH 8.0, with 50 mM NaCl, 5 mM MgCl2, 0.5 mM NADP+, 0.01 U isocitrate dehydrogenase (NADP+-dependent) from porcine heart (Sigma)]. The reaction was started by the addition of 45 μl buffer R2 (buffer R1 without NADP+ and isocitrate dehydrogenase, with 2.5 mM cis-aconitate). Activity was expressed as ΔΔE(340 nm)/Δt and was normalized for protein concentration. Isocitrate dehydrogenase activity was determined by following the reduction of NADP+ at 340 nm as described above. Five microliters of lysate was added to 45 μl buffer R3 (buffer R1 without isocitrate dehydrogenase). The reaction was initiated by the addition of 50 μl buffer R4 (buffer R3 without NADP+, with 5 mM isocitrate).
Succinate dehydrogenase activity in situ stain

Succinate dehydrogenase activity was evaluated on the basis of the succinate-dependent iodonitrotetrazolium chloride reduction. Prior to dissection the larvae were kept for 10 min in cold 1× PBS with 25 % (v/v) glycerol. Subsequently the cuticle and fat body of the larva (totally submerged in cold buffer) were carefully removed. The dissected tissue was briefly washed in cold isotonic buffer and was transferred on a coverslip. One hundred microliters of 50 mM Tris/HCl pH 7.5 with 1 mM rotenone, 1 mg/ml antimycin, 10 mM KCN, 25 mM azide, 0.1 % digitonin, and proteinase inhibitor was added to the sample. Tissues were completely submerged in this solution for 10 min. Following preincubation, 100 μl of 50 mM Tris/HCl pH 7.5 with 4 mM iodonitrotetrazolium chloride, 0.5 mM EDTA, 15 g/l CremophorEL (Sigma), and 50 mM succinate (for negative control without succinate) was added. Incubation was at room temperature for 10 min. The staining solution was removed with a pipette and filter paper and samples were imaged unfixed in 1× PBS with 25 % (v/v) glycerol. The reduction of iodonitrotetrazolium chloride leads to red, water-insoluble formazan.

Enhanced Prussian blue stain

The dissection was performed as described in the previous section. The samples were rinsed three times with 1× PBS and subsequently fixed and stained simultaneously in 4 % paraformaldehyde (v/v) and Perls’s solution [1 % K₄Fe(CN)₆ and 1 % HCl] for 30 min at room temperature. Enhancement with diaminobenzidine was performed as described in [45].

Results

Drosophila Hsc20 encodes a mitochondrial protein

An alignment between Drosophila and human Hsc20 protein sequences has revealed extended homology and 28 % identity between the homologue proteins of the two species [21]. The Drosophila Hsc20 gene is currently annotated in FlyBase as l(3)72Do, following a detailed genetic analysis of the polypene chromosome region 72A-D that revealed CG34246 as an essential gene [46]. Here we refer to CG34246 or l(3)72Do as Hsc20.

Recent genomic approaches under the Model Organism Encyclopedia of DNA Elements (modENCODE) project [47], supported by the National Human Genome Research Institute, have provided a developmental transcriptome in Drosophila melanogaster [48]. This work has revealed that Hsc20 contains four exons and is expressed at low levels throughout development (Fig. 1a) and in all tissues of larvae and adults (see http://flybase.org), consistent with a housekeeping role for a putative mitochondrial protein. The second intron is predicted to be 52 bp long and the third intron 60 bp long. Primers used to amplify the open reading frame of Hsc20 by reverse transcription PCR confirmed the difference of 112 bp between the PCR on the genomic template and the reverse transcription PCR product and also that Hsc20 is expressed in flies (Fig. 1b). A construct linking red fluorescent protein to the C-terminus of Hsc20 was transfected in HeLa cells to determine the subcellular localization of the protein, which was found to accumulate in mitochondria (Fig. 1c), a result that was expected on the basis of the characterization of the human protein and the conserved mitochondrial targeting sequence in the N-terminus of Drosophila Hsc20 [21].

Drosophila Hsc20 mutants show growth arrest and reduced ISC biogenesis

The two transgenic insertions used in this study were selected because their insertion points disrupt the first exon of Hsc20 at a position in the 5′ untranslated region (Fig. 1a). Consistent with what was shown previously for other transposons in this locus [46], both the Hsc20f2457 allele and the Hsc20f2457 allele are homozygous lethal and do not complement each other. The developmental time point of lethality was investigated by balancing the respective insertions to TM3, Kr-GFP, Sb, making heterozygous and homozygous genotypes recognizable through the presence or absence of fluorescence, respectively, throughout the organism’s development (Fig. 2a). The time point of lethality was identical for both alleles (shown only for the Hsc20f2457 allele); homozygous adult flies. In the experiment shown we counted 249 progeny flies, of which 183 (73 %) were heterozygous and 66 (27 %) were homozygous, of which 53 (22 %) died while trying to eclose from their pupal cases (Fig. 2b). Given that balancer chromosomes are homozygous lethal, a full rescue would result in 33 % of the Hsc20f2457 allele and 67 % of the Hsc20f2457 allele included UAS, which can be used to induce neighboring gene expression (from exons 2-4 that encode the Hsc20 open reading frame), we generated Actin-Gal4/Cyo; Hsc20f2457/TM3, Kr-GFP, Sb flies and looked for rescue (Fig. 2b). Given that balancer chromosomes are homozygous lethal, a full rescue would result in 33 % Hsc20f2457/TM3, Kr-GFP, Sb flies and 67 % Hsc20f2457/+ heterozygous adult flies. In the experiment shown we counted 249 progeny flies, of which 183 (73 %) were heterozygous and 66 (27 %) were homozygous, of which 53 (22 %) died while trying to eclose from their pupal cases (Fig. 2b, left panel) and only 13 (5 %) survived into adulthood. The Actin-Gal4/Cyo; Hsc20f2457/TM3, Kr-GFP, Sb genotype was also generated as a control, but no homozygous viable adults were obtained in this stock at any point. Hence, our rescue experiments show that the lethality associated with

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flies is due to a specific reduction in Hsc20 expression.

We collected third instar homozygous Hsc20 mutant larvae and their heterozygous sibling controls and quantified total aconitase and isocitrate dehydrogenase activities in lysates prepared from these. Both Hsc20\(^{f2457/f2457}\) and Hsc20\(^{c5018/c5018}\) larvae showed a marked decrease of aconitase activity to approximately 50% of the activity measured in the respective heterozygous flies (Fig. 3a, left panel). To verify that the loss of activity was due to a specific loss of ISCs in aconitase enzymes, we also tested isocitrate dehydrogenase, another housekeeping enzyme that does not depend on ISCs, in the same lysates and found no difference between the genotypes (Fig. 3a, right panel). We also tested for succinate dehydrogenase activity, this time employing an activity assay in dissected tissues of the larvae. We observed high activity in the anterior midgut of the intestine in Hsc20\(^{f2457/+}\) larvae, which was markedly reduced in Hsc20\(^{f2457/f2457}\) larvae (Fig. 3b). These results showed that ISC proteins are specifically impaired in Hsc20 mutant larvae.

\(Hsc20^{f2457/f2457}\) flies accumulate iron in mitochondria instead of in ferritin

To test the effect of defective ISC biogenesis on iron homeostasis, we recombined a GFP–ferritin trap line \([43, 49]\) with the \(\text{piggyBac}\) insertions in Hsc20. We then dissected intestines from \(Hsc20^{f2457}\), \(\text{Fer1HCHG188}/Hsc20^{f2457}\) third instar larvae and from \(Hsc20^{f2457}, \text{Fer1HCHG188}/\text{TM3, Sb}\) controls and observed ferritin under a fluorescence microscope. Ferritin accumulation in the iron region was unaffected between the two genotypes (Fig. 4a, middle panels); expression of ferritin in these cells was previously shown to be independent of iron \([49]\). In contrast, cells in the anterior and posterior midgut showed low, but readily detectable levels of ferritin accumulation in the heterozygous Hsc20 mutant larvae; these cells were devoid of ferritin in the homozygous Hsc20 mutants (Fig. 4a, top and bottom panels). Dietary iron induces ferritin in the anterior midgut of larvae \([43, 49]\), so we tested the response of Hsc20 mutants when grown on yeast supplemented with 1 mM ferric ammonium citrate. As expected, \(Hsc20^{f2457}, \text{Fer1HCHG188}/\text{Fer1HCHG188}/\text{TM3, Sb}\) larvae showed a clear induction of ferritin in the anterior midgut, whereas induction of ferritin in \(Hsc20^{f2457}, \text{Fer1HCHG188}/Hsc20^{f2457}\) intestines was compromised (Fig. 4b, top panels).

Ferritin induction in the anterior midgut serves as a store for iron absorbed by the diet \([43]\). We stained the intestines dissected from \(Hsc20^{f2457}\), \(\text{Fer1HCHG188}/Hsc20^{f2457}\) third instar larvae and from \(Hsc20^{f2457}, \text{Fer1HCHG188}/\text{TM3, Sb}\) controls and observed ferritin under a fluorescence microscope. Ferritin accumulation in the iron region was unaffected between the two genotypes (Fig. 4a, middle panels); expression of ferritin in these cells was previously shown to be independent of iron \([49]\). In contrast, cells in the anterior and posterior midgut showed low, but readily detectable levels of ferritin accumulation in the heterozygous Hsc20 mutant larvae; these cells were devoid of ferritin in the homozygous Hsc20 mutants (Fig. 4a, top and bottom panels). Dietary iron induces ferritin in the anterior midgut of larvae \([43, 49]\), so we tested the response of Hsc20 mutants when grown on yeast supplemented with 1 mM ferric ammonium citrate. As expected, \(Hsc20^{f2457}, \text{Fer1HCHG188}/\text{Fer1HCHG188}/\text{TM3, Sb}\) larvae showed a clear induction of ferritin in the anterior midgut, whereas induction of ferritin in \(Hsc20^{f2457}, \text{Fer1HCHG188}/Hsc20^{f2457}\) intestines was compromised (Fig. 4b, top panels).
magnification revealed a punctate pattern, which was not seen in Hsc20 heterozygous control stains (Fig. 4b, bottom panels).

Discussion

Hsc20 encodes a mitochondrial protein involved in ISC biogenesis

We have characterized two piggyBac insertions in the Hsc20 gene that result in reduced activities of classic ISC enzymatic activities and have shown that Hsc20 is a nuclear-encoded mitochondrial protein. Our results are largely similar to those obtained by other investigators who studied ubiquitous RNAi phenotypes of the dfh gene [32, 33] and therefore validate our conclusion that the Hsc20 mutants described here provide the first example of Drosophila mutants in ISC biogenesis.

Iron accumulation in mitochondria is characteristic of disrupted ISC biosynthesis

We observed a punctate staining for iron in Hsc20 mutant cells localized in the anterior intestine of larvae (Fig. 4b, lower panels). Accumulation of iron in mitochondria is a characteristic of Friedreich’s ataxia patients and is also observed in animal models of the disease [50] and yeast lacking Jac1 [24, 26]. Such pathologic iron is present in aggregate form, but is clearly distinct from ferritin iron [51]. Given the evidence accumulated in studies with yeast, mice models, and human patients [42], we believe that the punctate staining we have observed specifically in Hsc20 mutants could represent similar mitochondrial iron inclusions, suggesting these may also develop in the new Drosophila model of disrupted ISC biogenesis we have described. The phenotypes of reduced ISC activities, mitochondrial iron overload, and cytosolic iron deficiency are consistent with those reported for the human and yeast Hsc20 homologues.
For these reasons we suggest that Hsc20 should be considered as a candidate disease gene in humans.

Cell-type-specific effects in ferritin regulation in Hsc20 mutants

Intestinal ferritin regulation is complex and regulates both iron absorption and iron storage [43, 52–54]. The iron cells in the middle midgut express ferritin in a constitutive manner and independent of systemic iron concentrations [49]. Cells in the very posterior of the intestine appear to be specialized in zinc homeostasis, where ferritin may also have a specialized function that is not yet understood [55]. In this study, we have noted no changes in ferritin accumulation in the middle midgut in Hsc20 mutants (Fig. 4a, middle panels), consistent with the notion that iron homeostasis in these cells is specialized. The apparent downregulation of ferritin in cells of the anterior midgut (Fig. 4a, b, upper panels) may be explained by the activation of iron regulatory protein 1A as a result of defective ISC biogenesis and hence translational repression of ferritin [56, 57], or by mitochondrial iron accumulation causing a relative cytoplasmic iron deficiency. Whichever explanation holds (or both may be true), we note that not all cell types respond in the same way on genetic inactivation of Hsc20, calling for further investigations into the in vivo roles of the ISC biosynthetic machinery. The mutants described in this study should help expand in vivo studies.

An iron-related mitochondrial to nucleus signal may be relevant to the circadian clock

Drosophila melanogaster provides an elegant system for the parallel investigation of molecular, biochemical, cellular, physiological, and behavioral biology using genetic and environmental manipulations. The Hsc20 mutants we describe offer an alternative model to validate results obtained via RNAi experiments [32–36, 58]. Most other genes known from other systems to be involved in ISC biosynthesis are conserved in Drosophila, where they were implicated in the maintenance of circadian rhythms [58], representing the first proposed function of ISCs in the
regulation of animal behavior. At least one ISC-containing enzyme, dihydropyrimidine dehydrogenase, has been shown to have circadian rhythmic expression in the head of flies [59], but it is unknown which ISC protein may interact directly with the biological clock’s transcriptional feedback loops [60, 61]. The function of ISC proteins in regulating redox homeostatic mechanisms may help explain how the recently discovered free-running metabolic and redox cycles link to the cellular transcriptional and translational timekeeping networks [62, 63]. In plants, a retrograde signal from chloroplasts to nucleus signaling an iron deficiency has been suggested to affect the period length of the clock [64–66]. Such a signal could be the unknown chemical exported by the ABCB7 mitochondrial transporter [17, 67]. Owing to the lethality of Hsc20 mutants during their larval stage of development (Fig. 2a), we were unable to test whether loss of Hsc20 would disrupt the circadian clock. However, this lethality underscores the essential role of ISCs in animal growth.

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