Drosophila mitoferrin is essential for male fertility: evidence for a role of mitochondrial iron metabolism during spermatogenesis

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Abstract
Background: Mammals and Drosophila melanogaster share some striking similarities in spermatogenesis. Mitochondria in spermatids undergo dramatic morphological changes and syncytial spermatids are stripped from their cytoplasm and then individually wrapped by single membranes in an individualization process. In mammalian and fruit fly testis, components of the mitochondrial iron metabolism are expressed, but so far their function during spermatogenesis is unknown. Here we investigate the role of Drosophila mitoferrin (dmfrn), which is a mitochondrial carrier protein with an established role in the mitochondrial iron metabolism, during spermatogenesis.

Results: We found that P-element insertions into the 5'-untranslated region of the dmfrn gene cause recessive male sterility, which was rescued by a fluorescently tagged transgenic dmfrn genomic construct (dmfrnvenus). Testes of mutant homozygous dmfrnSH115 flies were either small with unorganized content or contained some partially elongated spermatids, or testes were of normal size but lacked mature sperm. Testis squashes indicated that spermatid elongation was defective and electron micrographs showed mitochondrial defects in elongated spermatids and indicated failed individualization. Using a LacZ reporter and the dmfrnvenus transgene, we found that dmfrn expression in testes was highest in spermatids, coinciding with the stages that showed defects in the mutants. Dmfrn-venus protein accumulated in mitochondrial derivatives of spermatids, where it remained until most of it was stripped off during individualization and disposed of in waste bags. Male sterility in flies with the hypomorph alleles dmfrnBG00456 and dmfrnEY01302 over the deletion Df(3R)ED6277 was increased by dietary iron chelation and suppressed by iron supplementation of the food, while male sterility of dmfrnSH115/Df(3R)ED6277 flies was not affected by food iron levels.

Conclusions: In this work, we show that mutations in the Drosophila mitoferrin gene result in male sterility caused by developmental defects. From the sensitivity of the hypomorph mutants to low food iron levels we conclude that mitochondrial iron is essential for spermatogenesis. This is the first time that a link between the mitochondrial iron metabolism and spermatogenesis has been shown. Furthermore, due to the similar expression patterns of some mitochondrial iron metabolism genes in Drosophila and mammals, it is likely that our results are applicable for mammals as well.

Background
Iron is an essential micronutrient for almost all organisms and is used as a co-factor for many enzymes involved in redox-reactions. Its reactivity with hydrogen-peroxide also bears the potential to promote the formation of reactive oxygen species via the Fenton reaction. Reactive oxygen species in turn result in, protein, lipid and DNA damage that can lead to cellular dysfunction and damage to organs. Consequently, free iron levels must be kept at a minimum, while enough iron must be provided to processes that depend on it (reviewed in [1,2]).

Mitochondria are the sites of iron-insertion into protoporphyrin IX [2] and iron-sulfur cluster (ISC) biosynthesis [3,4] within eukaryotic cells and are, therefore, the subcellular compartments with the highest requirement for iron. Transport of iron into mitochondria, is facili-
tated by the mitochondrial carrier proteins Mrs3p and Mrs4p (Mrs3/4p) [6,7] in yeast. MRS3/4 genes have been shown to genetically interact with frataxin [7] in the delivery of iron to heme [8] and ISC synthesis [9] in mitochondria. At least in yeast, another less effective mitochondrial iron transport mechanism seems to exist, as MRS3/4 mutants only manifest phenotypes at low iron conditions [10].

In vertebrates two paralog genes that are homologs to MRS3/4 exist. Mitoferrin1, is mainly expressed in erythropoietic tissues and the frascati mutations in the zebrafish result in hemoglobinization defects, anemia and lethality [11]. Both mitoferrin1 and mitoferrin2 can rescue yeast MRS3/4 double mutants, indicating a similar function. Only ectopic expression of mitoferrin1 can rescue frascati mutants [11] and it was recently shown that mitoferrin1 protein, but not mitoferrin2, accumulates in erythropoietic cells at amounts that can meet the need of mitochondria for iron in these cells [12].

In a previous study we found that Drosophila melanogaster and other invertebrates (i.e., sea urchin, Caenorhabditis elegans, bee, wasp, mosquito and flour beetle) have only one mitoferrin gene, which is most likely a functional homolog of vertebrate mitoferrin2 as invertebrates lack erythropoiesis [13]. Study of Drosophila mitoferrin (dmfrn) in insect cell culture showed that its dysregulation affects cellular iron homeostasis through the iron-sulfur cluster synthesis pathway [13].

Mitochondria play an important role during spermatogenesis. For example, defects in caspase activation involving the spermatogenesis-specific cytochrome gene cyt-c-d [14] or defects in mitochondrial fusion processes, involving the fuzzy onions gene product [15], result in male sterility with defects during spermatogenesis. Drosophila melanogaster testes are 2 mm long terminally blind tubes. Spermatogenesis starts at the apical tip where stem-cell divisions give rise to germ cells [16]. Each germ cell is contained in a cyst of two somatic cyst cells [17] and undergoes four mitotic divisions, resulting in 64 syncytical spermatids after meiosis [18]. Mitochondrial fusion processes in spermatids result in two giant mitochondrial derivatives per spermatid that furl up to form the nebenkern. During elongation, the mitochondrial derivatives unfurl along the flagellar axoneme. As the individualization complex progresses along the length of the spermatids, each spermatid is wrapped in its own membrane, the minor mitochondrial derivative is depleted of most of its material, and other organelles and most of the cytoplasm are removed from spermatids and accumulate in the cystic bulge, which is cast off at the end of the spermatids as a waste bag [18]. After coiling, individualized spermatids are released from their cyst and stored as mature sperm in the seminal vesicle.

Here we report on a function of dmfrn during spermatogenesis and characterize its expression in different fly tissues as well as within testis. We show for the first time that the mitochondrial iron metabolism is essential during spermatogenesis.

**Results**

**P-element insertion P(lacW)dmfrnSH115 in the 5’-untranslated region of dmfrn results in male sterility**

Previously we have identified dmfrn (CG4963), the only Drosophila homolog of yeast Mrs3/4 and vertebrate mitoferrin2, and studied its role in cellular iron homeostasis in cell culture [13]. Little is known about the role of mitoferrin2 in the whole organism.

To study dmfrn, we obtained four publicly available mutant alleles. Three are due to P-element insertions in the 5’ untranslated region (5’ UTR) of the dmfrn gene and one is a deficiency where dmfrn and a small part of the region in its proximity are deleted (Df(3R)ED6277; Figure 1A).

The insertion site of P(lacW)][(3)SH115SH115 is downstream of the putative transcriptional start (about 252 bp) but upstream of the start of the dmfrn-coding sequence (Figure 1A). It was recovered during a screen for recessive lethal genes [19], which would be in agreement with iron-sulfur clusters being essential co-factors [3] and the proposed general function of mitoferrin2 in mitochondrial iron transport in non-erythroid tissues [11]. P-elements P(GT1)dmfrnB00456 and P(Epgy2)dmfrnEY01302 are both located closer to the putative transcriptional start of dmfrn (about 20 and 40 bp downstream, respectively, Figure 1A) and no phenotypes caused by the insertions themselves have been reported. P(Epgy2)dmfrnEY01302 contains an UAS sequence upstream of dmfrn and has been used in a gain of function screen, where overexpression of dmfrn in developing muscle apodemes caused muscle misdevelopment [20].

While cleaning the fly lines from background mutations by outcrossing them with wild type flies (strain w1118), lethality of SH115[(3)SH115 flies was lost after only three generations. Therefore, it was unlikely that the reported lethality was caused by allele SH115[(3)SH115 but by another unmarked mutation instead. Consequently, the allele SH115[(3)SH115 should be referred to as dmfrnSH115.

To our surprise we failed to establish a homozygous stock of dmfrnSH115 flies, as male flies were recessive sterile and sterility was completely penetrant. Female dmfrnSH115 flies were fertile (100% fertile; n = 20), while male recessive sterility persisted even after outcrossing dmfrnSH115 flies for 13 generations to w1118 flies. To confirm that the allele dmfrnSH115 was indeed responsible for male sterility, a P-element excision screen, in which the P-
element was re-mobilized, was carried out [21]. Independent lines of flies that had lost the marker for the P-element were established and analyzed by PCR for precise and imprecise excisions (i.e., P-element leftovers or genomic deletions) (Table 1). All precise excisions in trans to dmfrnSH115 rescued the recessive male sterility phenotype (Table 1). These results show that P{lacW}dmfrn SH115 causes male sterility.

We also analyzed fertility in male flies that carried dmfrnSH115 in trans to Df(3R)ED6277 or the small deletion dmfrnDf13 (Figure 1A), which was recovered during the hop-out assay, as well as flies that carried dmfrnDf13 in trans to Df(3R)ED6277 or were homozygous for Df(3R)ED6277. All of these combinations of dmfrn mutations resulted in male sterility (Table 2). To further support the role of dmfrn in male fertility and to rule out that P{lacW}dmfrnSH115 interfered with enhancer or repressor elements of other nearby genes, and thereby resulted in male sterility, we performed a genomic rescue experiment using a transgene fly line, which contains the gene region of dmfrn including the intergenic region between dmfrn and Gp93. The stop codon of dmfrn was substituted by the coding sequence of the fluorescent marker venus, resulting in the expression of a C-terminally venus-tagged dmfrn protein. The 5’ and 3’ inverted repeats of the P-element used for generation of transgene flies by P-element transposition are indicated by the black boxes.

**Figure 1** The Drosophila mitoferrin (dmfrn) gene region and the genomic rescue construct dmfrnvenus. **A:** P-element insertion sites and deletions in dmfrn. The gene dmfrn is encoded on the (-)strand and the coding sequence of lacZ in P-element P{lacW}dmfrnSH115 is encoded on the (+) strand as well (see Additional file 1 Figure A1A and A1D). **B:** The genomic rescue construct dmfrnvenus contains the gene region of dmfrn including the intergenic region between dmfrn and Gp93. The stop codon of dmfrn was substituted by the coding sequence of the fluorescent marker venus, resulting in the expression of a C-terminally venus-tagged dmfrn protein. The 5’ and 3’ inverted repeats of the P-element used for generation of transgene flies by P-element transposition are indicated by the black boxes.

| Hop-out class         | Total number of lines | Homozygous fertile | Hop-out/SH115 fertile |
|-----------------------|-----------------------|---------------------|----------------------|
| precise excision      | 11                    | 10                  | 11                   |
| transposon leftovers  | 7                     | 1                   | 1                    |
| deletion              | 3                     | n.a.*               | n.a.                 |

*the lines exhibited different degrees of lethality
spermatogenesis. that is a sign for cytokinesis defects [23]. This indicates normal sized nuclei (see Additional file 1 Figure A2), flies showed larger Nebenkerns associated with several males were crossed to female Df(3R)ED6277/TM6c flies, and the small or the large deletion on the third chromosome, dmfrnvenusB32 on the second chromosome, and either the three P-element insertions in trans to Df(3R)ED6277 causes lethality in addition to sterility. Flies with any of Df(3R)ED6277 adult flies was lower than expected (6% sterility of homozygous Df(3R)ED6277 males is likely caused by the deletion of one of the three other genes affected by the larger deletion. According to the FlyAtlas [22], the uncharacterized gene CG5514 is expressed in nerve tissue, ovaries and testes and is therefore the best candidate for male sterility of Df(3R)ED6277 males is probably caused by the deletion of one of the three other genes affected by the larger deletion. According to the FlyAtlas [22], the uncharacterized gene CG5514 is expressed in nerve tissue, ovaries and testes and is therefore the best candidate for male sterility of Df(3R)ED6277 males (i.e., cell proliferation, growth and morphogenesis) and the male sterility phenotype of dmfrnSH115 flies was completely penetrant, we continued investigating the role of dmfrn during spermatogenesis. 

Table 2: dmfrnvenusB32 rescues male sterility of homzygous dmfrnSH115 as well as transheterozygote dmfrnSH115/DmfrnDf13, dmfrnSH115/Df(R3)ED6277 and dmfrnDf13/Df(R3)ED6277 male flies but not homzygous Df(R3)ED6277 male flies. 

| Genotype | % fertile flies |
|----------|----------------|
| without rescue construct | |
| SM1 Cy/+; dmfrnSH115 | 0 (n = 37) |
| SM1 Cy/+; dmfrnSH115/dmfrnDf13 | 0 (n = 50) |
| SM1 Cy/+; dmfrnSH115/Df(3R)ED6277 | 0 (n = 30) |
| SM1 Cy/+; Df(3R)ED6277 | 0 (n = 10) |
| SM1 Cy/+; Df(3R)ED6277 | 0 (n = 17) |
| with rescue construct | |
| dmfrnvenusB32/; dmfrnSH115 | 97 (n = 33) |
| dmfrnvenusB32/; dmfrnSH115/dmfrnDf13 | 100 (n = 25) |
| dmfrnvenusB32/; dmfrnSH115/Df(3R)ED6277 | 97 (n = 30) |
| dmfrnvenusB32/; dmfrnDf13/Df(3R)ED6277 | 100 (n = 37) |
| dmfrnvenusB32/; Df(3R)ED6277 | 0 (n = 22) |

...rility of homzygous dmfrnSH115 flies as well as sterility of dmfrnSH115/dmfrnDf13 flies, dmfrnSH115/Df(3R)ED6277 flies, and dmfrnDf13/Df(3R)ED6277 flies was rescued by the transgene dmfrnvenusB32 construct (Table 2). However, male sterility of homzygous Df(3R)ED6277 males was not rescued by dmfrnvenusB32 (Table 2), indicating that the sterility of homzygous Df(3R)ED6277 males is likely caused by the deletion of one of the three other genes affected by the larger deletion. According to the FlyAtlas [22], the uncharacterized gene CG5514 is expressed in nerve tissue, ovaries and testes and is therefore the best candidate for male sterility of Df(3R)ED6277 flies. Inspection of testes squashes in these flies showed larger Nebenkerns associated with several normal sized nuclei (see Additional file 1 Figure A2), which is a sign for cytokinesis defects [23]. This indicates that CG5514 might be involved in cytokinesis during spermatogenesis. 

The fact that the emergence of homzygous Df(3R)ED6277 adult flies was lower than expected (6% instead of ~33%; Figure 2A), indicates that Df(3R)ED6277 causes lethality in addition to sterility. Flies with any of the three P-element insertions in trans to Df(3R)ED6277 did not show any obvious signs of lethality (Figure 2B). Male flies with the genomic rescue construct dmfrnvenusB32 on the second chromosome, and either the small or the large deletion on the third chromosome, were crossed to female Df(3R)ED6277/TM6c flies, and offspring were scored. The fractions of dmfrnDf13/Df(3R)ED6277 and homzygous Df(3R)ED6277 flies that carried the rescue construct (27% and 18%, respectively) were larger than the fractions of flies without the rescue construct (13% and 12%, respectively) (Figure 2A). These results show that the deletion of dmfrn results in partial lethality, indicating an essential role of dmfrn during development to adulthood.

As spermatogenesis uses many processes that are also needed during the normal development of an organism (i.e., cell proliferation, growth and morphogenesis) and the male sterility phenotype of dmfrnSH115 flies was completely penetrant, we continued investigating the role of dmfrn during spermatogenesis.

**dmfrnSH115 causes elongation defects during spermatogenesis**

Male sterility can result from defects in mating behavior, abnormal anatomy of the sexual organs or defects during spermatogenesis [24]. Since homzygous dmfrnSH115 males were observed mating, behavioral abnormalities were ruled out as the cause of male sterility. Dissection of male flies revealed that the testes of homzygous dmfrnSH115 flies exhibited a defect during spermatogenesis of variable intensity resulting in the absence of mature sperm, whereas heterozygous testes looked normal and contained motile mature sperm (Figure 3A arrow head). The severity of the spermatogenesis defect in homzygous dmfrnSH115 flies ranged from testes that lacked properly elongated spermatids to those that looked almost like wild type (WT) testes but lacked mature sperm (Figure 3B to 3D shows some examples).

By analyzing testes squashes we found that dmfrnSH115 spermatocytes (Figure 3F) and onion stage spermatids (Figure 3H) looked normal, whereas later stages of spermatids showed different defects. We found signs for delayed spermatid elongation (Figure 3J and 3K). Often, we observed abundant white structures of unknown origin in spermatid bundles (Figure 3L and 3L'). Sometimes mitochondrial derivatives of elongating and elongated spermatids had bulby protrusions (Figure 3N and 3N' arrow head) that are reminiscent of unelongated or improperly elongated mitochondrial derivatives. We also observed elongated spermatids that appeared normal, but never mature sperm. This is supported by the finding that all dmfrnSH115 males were completely sterile. All of these results are indicative of an elongation defect in dmfrnSH115 testes.

At the ultrastructural level, elongated spermatids of dmfrnSH115 testes exhibited great morphological defects compared to those of WT testes. Spermatid cysts were unorganized (compare Figure 4A and 4C to Figure 4D and 4I). In testes of dmfrnSH115 flies, the major and minor mitochondrial derivatives, associated with axonemes...
Figure 2 Development of dmfrn mutants to adulthood. Female Df(3R)ED6277/TM6c flies were crossed to male flies of the indicated genotype and allowed to lay eggs for three days. Numbers in parentheses show the total number of flies assayed per genotype. (A) Eclosed adult flies were collected and genotyped according to their genotypic markers. (B) Eclosed flies were sexed and genotyped according to their genotypic markers during an eclosion period of seven days. Male and female balanced: flies heterozygous for dmfrn mutations. Male and female dmfrn: the indicated dmfrn mutation in trans to Df(3R)ED6277.
Figure 3 Testes of dmfrnSH115 flies show defects during spermatogenesis. Testis from a heterozygous dmfrnSH115/TM6c fly with mature motile sperm (A arrow head). Testes from homozygous dmfrnSH115 flies lack mature sperm, can be smaller (B) than WT, often have fewer elongated spermatids (C and D) or can look similar to WT testes (D). Phase contrast of testes squashes of w1118 (E, G, I and M) and dmfrnSH115 flies (F, H, J, K, L, L', N and N'). Spermatocytes (F) and onion stage spermatids (H) of dmfrnSH115 testis did not show any obvious defects and are indistinguishable from WT spermatocytes (E) and onion stage spermatids (G). Early elongating spermatids of dmfrnSH115 testis show signs of delayed elongation (J and K; the arrow indicates the dark spot on the nucleus, which is characteristic for elongating spermatids, as can be seen in WT (I)). Spermatids of dmfrnSH115 testes frequently contained white spherical objects (L and L'). Membrane blebbing was observed on mitochondrial derivatives of elongating spermatids of dmfrnSH115 testes (N and N' arrow) but not on WT testes (M, arrow).
Figure 4  Spermatids from *dmfrn^SH115* flies show defects in the maturation of the mitochondrial derivatives. Transmission electron micrographs of ultra thin sections of tests from WT flies (A-C) and *dmfrn^SH115* flies (D-I). (A) Overview and (B) close-up of pre-individualization spermatids, showing the symmetric distribution of the spermatids within the cyst. The major mitochondrial derivative can easily be distinguished from the minor mitochondrial derivative by the accumulation of the electron dense stain. (C) Overview of individualized spermatids, which shows paracrystalline symmetry, a clearly visible major mitochondrial derivative and depletion of cytoplasm. (D) Overview and (E) close-up of *dmfrn^SH115* pre-individualization spermatids. No symmetry of the spermatids can be observed and accumulation of the paracrystalline structure to only a few major mitochondrial derivatives can be seen. (F and H) Overview and (G and I) close up of spermatids from *dmfrn^SH115* flies. Several axonemes are within the same spermatids and the accumulation of the paracrystalline structure within mitochondria is very heterogeneous.
were impossible to distinguish from one another in many cases. Often, one axoneme was associated with two mitochondrial derivatives of similar size, and both had accumulated the paracrystalline structure (Figure 4G), which is normally typical for the major mitochondrial derivative [25,18]. In some cases, one such mitochondrial derivative was extremely enlarged and contained heterogeneous accumulations of the paracrystalline structure (Figure 4G). Furthermore, in the same cyst the number of spermatids wrapped in the same membrane was not constant (Figure 4H), indicating that if individualization occurred, it was defective.

Defects during spermatogenesis can either be primary defects, which are a direct consequence of a mutation or they can be secondary defects, which are the result of primary defects [23]. To find out whether possible defects during individualization, as indicated by the above TEM results, were preceded by earlier defects that are independent of the elongation defects that we observed under phase contrast, we analyzed whole mount testis of dmfrnSH115 flies by confocal laser scanning microscopy. Nuclei and f-actin were stained with DAPI and rhodamin phalloidin, respectively. In WT testes, nuclei of elongated spermatids were needle-shaped and located at the base of the testis in parallelly packed bundles (Figure 5A, DAPI) and individualization complexes present at different positions of elongated spermatids were apparent from well organized actin-cones (Figure 5A, RhoPha). Even though nuclei of spermatids from dmfrnSH115 testes were needle-shaped, they were often scattered over large areas of the length of the testes (Figure 5B, DAPI) or formed fuzzy bundles (Figure 5C, DAPI). Despite the presence of parallel organized nuclei in some mutant spermatid bundles, we did not observe actin-cones in the mutant testes we analyzed (Figure 5B and 5C, DAPI). However, in one testis we observed the association of f-actin with nuclei, which might be indicative for the formation of an individualization complex (Figure 5C). So, even though we could not see any actin cones, it may not be ruled out that some spermatids did form individualization complexes and underwent partial individualization. All defects observed in dmfrnSH115 testes were rescued in testes of dmfrnYenus382/+; dmfrnSH115 flies (Figure 5D).

A parallel organization of the nuclei at the start of individualization is required for the successful assembly of functional individualization complexes. From the above results, it is therefore likely that the defects observed by TEM in dmfrnSH115 testes are the result of the elongation defects.

dmfrn is ubiquitously expressed with slightly higher expression in testes

Mitochondrial ferritin, an iron storage protein, is most abundantly expressed in testes of mice [26,27] and flies [28], indicating that the mitochondrial iron metabolism, in particular, might play a role during spermatogenesis in insects and mammals.

According to the FlyAtlas [22], dmfrn expression is lowest in testes, which was rather unexpected in light of our findings. This prompted us to verify dmfrn expression in different tissues by RT-RT PCR. We isolated RNA from heads, thoraxes, guts, malpighian tubules and testes of 2-3 days old virgin male flies and analyzed the expression levels of dmfrn and other iron metabolism related genes (i.e., frataxin homolog (fh), Fer1HCH, Fer2LCH and Fer3HCH) as well as the house keeping gene RP49.

Mitochondrial ferritin (Fer3HCH) was expressed at extremely low levels in all tissues except for testes where its message was about eight times more abundant than in whole fly homogenates (Figure 6). This expression pattern is in agreement with that of a previous report [28] and is similar to the FlyAtlas expression data (about 10 times more transcript in testes, compared to whole flies of both sexes).

Transcripts of Fer1HCH and Fer2LCH were enriched about twofold in the gut and malpighian tubules but not in testes (Figure 6), which again is similar to the pattern reported in the FlyAtlas. Ferritins of Drosophila are involved in iron storage and iron transport (for a review on insect iron metabolism see [29]), and accumulate in the iron region of the midgut of iron loaded flies [30]. Higher expression of ferritins in the gut could therefore either be an indication for the mobilization of food iron to other tissues or iron storage.

The expression patterns of dmfrn and fh in our analysis (Figure 6) diverged from the FlyAtlas data. In all tissues, except for testes, both were expressed at levels similar to that of whole flies. In testes, message abundance of dmfrn and fh were about two-fold higher (Figure 6).

dmfrn is expressed at increased levels in spermatids

The above mentioned results indicate that dmfrn expression in testes is higher than in other tissues. Testes contain both somatic cells; i.e, cyst cells and cells of the testis sheath, as well as germline cells of different developmental stages. Mitochondrial aggregation and formation of the giant mitochondrial derivatives take place in spermatids [24] and since we observed spermatid elongation defects, we suspected that mitoferrin expression could occur before or during this stage.

P{lacW}dmfrnSH115 contains the coding sequence (CDS) of the β-galactosidase gene (lacZ) [31] on the same strand as dmfrn (Figure 1A and see Additional file 1 Figure A1) allowing the visualization of the expression pattern of dmfrn in testes through X-gal staining. Blue stain was not detectable in testis of w1118 flies, which were used as a negative control (Figure 7A left). In testis from heterozygous dmfrnSH115 flies, blue stain accumulated in
Figure 5 Spermatid elongation defects in testis of *dmfrn*SH115 flies. Nuclei were stained with DAPI (blue) and actin filaments were stained with rhodamine phalloidin (red). (A) Nuclei of elongated spermatids in WT testis are needle shaped and arranged in parallel (arrow). Individualization complexes (arrow heads) are visible as parallel arranged cone-shaped structures. (B) Nuclei of elongated spermatids in testis of *dmfrn*SH115 flies are needle shaped but scattered (arrows). Individualization complexes were not observed. (C) Higher magnification of the base of a testis from *dmfrn*SH115 flies, showing the scattered, but needle shaped nuclei of elongated spermatids. Two nuclei bundles are associated with f-actin structures (arrows), which might be newly forming individualization complexes. (D) Parallel, needle shaped nuclei (arrows) and individualization complexes (arrow heads) are observed in *dmfrnvenusB32/+; dmfrn*SH115 flies. Images were acquired by confocal laser scanning microscopy and represent optical slices through testes.
spermatids and was absent from spermatocytes and the rest of the genitalia (Figure 7A middle). X-gal staining of small testis from homozygous dmfrnSH115 flies showed the same pattern and only a few spermatids were elongated to a small degree (Figure 7A right).

To verify the expression pattern of dmfrn and to be able to study the localization of dmfrn protein during spermatogenesis in further detail, we examined testes from dmfrnvenusB32 flies under the fluorescence microscope. First, we made sure that the signal of dmfrn-venus protein was clearly discernible from autofluorescence of testes by using w1118 flies as a negative control (Figure 7B left). A clear signal of dmfrn-venus protein was visible in elongated spermatids (Figure 7B right). This expression pattern is similar to the expression pattern of dmfrn obtained through the expression of lacZ, confirming that dmfrn is expressed late during spermatogenesis. Using confocal microscopy with increased gain, we were also able to detect dmfrn-venus protein in spermatocytes and the testis sheath (see Additional file 1 Figure 3).

At higher magnification, we observed that dmfrn-venus protein accumulated in elongated spermatids, the region of spermatid individualization, and that a large fraction was disposed of in waste bags (Figure 8A and 8B). Closer inspection of whole mount dmfrnvenusB32 testes, using confocal microscopy, confirmed these observations (Figure 8C to 8E) and showed that dmfrn-venus protein abundance was increased in nebenkerns of onion stage spermatids and in elongated spermatids (Figure 8C). During spermatid individualization, dmfrn-venus accumulated in mitochondrial whorls in front of the actin cones of individualization complexes (Figure 8D). At the end of spermatid individualization, the bulk of dmfrn-venus had accumulated in cystic bulges and ended up in waste bags (Figure 8E).

Male fertility of hypomorph dmfrn mutants depends on food iron levels

Yeast MRS3/4 double deletions only cause a growth defect on low iron medium [10]. We were therefore interested to see whether food iron levels had any effect on fertility of the mfrnSH115 mutant. We also wanted to test if the fertility of flies with the two other P-element insertions, which could be kept as homozygous stocks on normal food, were influenced by iron availability.

To be able to quantify and compare fertility of the different P-element mutants, we wanted to use a genetic
background that was as similar as possible. Therefore, we crossed male dmfrnSH115/TM6c or dmfrnEY01302/TM3 or dmfrnBG00456/TM6c flies with female Df(3R)ED6277/TM6c flies. Eggs were laid on low iron food (food containing the iron chelator BPS), normal food and high iron food (~2.5 mM Fe³⁺ as ferric ammonium citrate (FAC)) to allow development of offspring under these different iron conditions. Males that carried the respective mutant allele over Df(3R)ED6277 were collected and fertility was quantified.

Df(3R)ED6277/TM6c males raised on low iron food were completely fertile (Figure 9A), showing that iron starvation by itself did not cause male sterility. Male sterility on normal food was low in dmfrnBG00456/Df(3R)ED6277 flies, higher in dmfrnEY01302/Df(3R)ED6277 flies and complete in dmfrnSH115/Df(3R)ED6277 flies (Figure 9A). Supplementation of food with iron increased fertility of dmfrnEY01302/Df(3R)ED6277 flies strongly, while low iron conditions reduced fertility of dmfrnBG00456/Df(3R)ED6277 and dmfrnEY01302/Df(3R)ED6277 males drastically (Figure 9A). dmfrnSH115/Df(3R)ED6277 male flies were completely sterile, regardless of the food they were raised on (Figure 9A).

We also observed testes of the different P-element mutants raised on low iron food. Testes of TM6c/Df(3R)ED6277 flies, which were used as a control, looked like normal WT testes with abundant elongated spermatids and mature motile sperm in the seminal vesicle (Figure 9B), showing again, that low iron levels alone do not cause defects during spermatogenesis. Testes of dmfrnBG00456/Df(3R)ED6277 and dmfrnEY01302/Df(3R)ED6277 flies contained very few elongated spermatids (Figure 9C and 9D and Figure 9E and 9F, respectively), whereas mfrnSH115/Df(3R)ED6277 testes were very small and did not contain elongated spermatids (Figure 9G). This, in turn, shows again that dmfrnSH115, which is further downstream in the 5' UTR of dmfrn, causes the strongest phenotype.

From these results, and the well established role of mitoferrins in the mitochondrial iron metabolism, we conclude that dmfrn and the mitochondrial iron metabolism are essential for spermatogenesis.

**Discussion**

The yeast homologs of dmfrn and frataxin homolog (fh) have been shown to genetically interact in yeast during iron-sulfur cluster (ISC) biosynthesis [9] and heme biosynthesis [8]. Previously, we have shown that dmfrn rescues yeast MRS3/4 double mutants and that its overexpression alters cellular iron homeostasis [13]. In the current study we show that the mitochondrial iron metabolism plays a role during spermatogenesis for the first time directly, through the male sterility phenotypes caused by P-element insertions into dmfrn and the dependence of the hypomorph mutant on dietary iron, and indirectly, through the expression of fh and dmfrn in testes. This is even further supported by the high expression level of mitochondrial ferritin in testis (our results and [28]). The fact that frataxin, mitochondrial ferritin and dmfrn/mitoferrin2 are all expressed at higher levels in testes of Drosophila and mammals [32,26,27,11,33], indicates that the involvement of the mitochondrial iron metabolism in spermatogenesis is very likely conserved from insects to mammals.

Early studies of mammalian spermatogenesis indicated a nutritional function of iron during spermatogenesis. In human seminal plasma, levels of transferrin, an ubiquitous iron transport protein in mammals, are correlated with sperm abundance [34]. Furthermore, transferrin is produced in Sertoli cells (“nurse cells”) of mammalian testes and delivers iron to germinal cells [35-37]. Defects in
Figure 8 Localization of dmfrn-venus protein in testis using conventional microscopy (A and B) and confocal laser scanning microscopy (C-E). (A) Phase contrast (pha. contr.) and fluorescence (venus, green) microscopy of a testis from a dmfrnvenusB32 fly. dmfrn-venus protein accumulates in elongated spermatids and waste bags. (B) Higher magnification of the area indicated in (A), showing the accumulation of dmfrn-venus in waste bags. (C-E) Confocal laser scanning microscope images of dmfrnvenusB32 expression in spermatids. (C) Expression of dmfrnvenusB32 (venus, green) in whole mount testis. Mitochondria stained with MitoTracker Deep Red 633 (MitoTracker, red). Arrow: onion stage spermatids; arrow head: elongated spermatids. (D) Localization of dmfrn-venus inside the cystic bulge at the individualization complex. dmfrn-venus (venus, green) accumulates in mitochondrial whorls in front of the actin cones (RhoPha, red) of the individualization complex. (E) After individualization, dmfrn-venus (venus, green) accumulates in waste bags.
the mitochondrial iron metabolism, especially the iron-sulfur cluster synthesis pathway, result in cellular iron accumulation [38-40]. Increased iron uptake and accumulation in yeast MRS3/4 mutants have been shown [41] and might occur in dmfrn mutants. It has been shown that injection of iron into testes of rats results in sterility and tissue degeneration [42], and similar experiments with other metals do suggest that this might be a general effect of heavy metals [43]. However, the fact that nutritional iron loading of dmfrn mutant flies rescued the weaker male sterility phenotypes and that iron starvation enhanced these phenotypes, indicates that the spermatogenesis defects in the testis of dmfrn mutants is not the result of cellular iron overload. We interpret our results as further support for a nutritional function of iron during spermatogenesis. This would also be in agreement with the large variety within the testis development we observed (ranging from very small to WT-like testis that lack mature sperm).

The growth defect of yeast MRS3/4 double deletions develops only under iron limiting conditions, and it has been reasoned that other, still unidentified transporters with lower iron affinity could compensate for the lack in Mrs3/4p under iron replete conditions [10,6]. Deletion of dmfrn results in partial lethality, whereas flies with the P-element insertions in the 5’ UTR of dmfrn are viable. Therefore, we suggest that there is residual dmfrn expression in the P-element insertion lines. In those lines with P-elements integrated closest to the putative transcriptional start site of dmfrn, expression might be high enough to allow nutritional iron loading to compensate for lower dmfrn expression, while dmfrn expression in dmfrnSH115 testes would be insufficient to sustain spermatogenesis, even under iron loading conditions. Testes are heterogeneous microenvironments and the germ cells are contained within a pair of cyst cells during all stages of development, and the function of cyst cells in Drosophila is poorly understood. Therefore, it could very likely be that access of germ cells to iron is controlled by cyst cells, and that even iron loading of dmfrnSH115 flies cannot provide enough iron for a low affinity transporter to compensate for the lack of dmfrn in dmfrnSH115 testes.

Several properties and phenomena of spermatogenesis are likely to rely on mitochondrial iron metabolism: (i)
Mitochondria of spermatids aggregate to form the giant nebenkerns through fusion processes that depend on mitochondrial activity [44], which in turn depends on the activity of the respiratory chain. Several complexes of the respiratory chain contain heme or ISC or both as prosthetic groups, linking energy metabolism directly with the mitochondrial iron metabolism. Insufficient dmfrn expression could lead to defects in the energy metabolism of the giant mitochondria and interfere with mitochondrial dynamics. (ii) Spermatids undergo dramatic morphological changes as they elongate to a length of almost 2 mm. This process is most likely very energy craving and, therefore, well functioning electron transport chains should be essential. If the energy metabolism is corrupted, elongation is likely to stop or slow down. (iii) Spermatids undergo an apoptosis-like processes during their individualization. The testis specific variant of the heme protein cytochrome c, encoded by the gene cyt-c-d, has been shown to be essential for spermatid individualization [45,14] and its function might be sensitive to heme deficiency. Even though we observed individualization defects that could hint to defects in the apoptosis-like process in spermatids of dmfrn mutants, it cannot be ruled out that preceding defects during spermatid elongation are the underlying cause.

The exact functions of the major and minor mitochondrial derivatives of insect sperm are not clear and several different hypotheses exist. Mitochondrial derivatives may be extremely efficient mitochondria or are degenerated mitochondria or modulate the undulation of the sperm tail in a species specific manner [25]. During spermatid individualization, a large part of the minor mitochondrial derivative is removed from spermatids tails and is disposed of in waste bags [18] and we found that a large fraction of dmfrn follows this portion of mitochondria. As the sperm tail is stripped from all of its organelles, except for the remaining part of the mitochondrial derivatives, mitochondrial transport is very likely to become obsolete. On the other hand, remaining mitochondrial carriers could clean the cytoplasm from left-over substrates. In Drosophila, ferritin resides in the endoplasmatic reticulum (ER) and can be secreted [46]. Using testes of Fer1HCHG188 flies that express GFP-tagged Ferritin Heavy Chain Homolog protein [30], we were able to identify ferritin in close proximity to mitochondrial whorls and its accumulation in waste bags (see Additional file 1 Figure A4). The close proximity of the ER to mitochondrial whorls could be an indication of iron transfer from the Fer1HCH/FerLCH pool to mitochondria to maintain a functional respiratory chain and active cytochrome-c-d.

A recently published article reports that the copper transporter Ctr1C in Drosophila, is essential for male fertility in a Ctr1B mutant background [47]. Furthermore, Ctr1C locates to the cytoplasmic membrane and is expressed in spermatids and elongating spermatids [47], indicating that metals, in general, play an important role during spermatogenesis.

Conclusions
From our findings we conclude, that Drosophila mitoferrin and the mitochondrial iron metabolism are essential during spermatogenesis. Drosophila and mammalian spermatogenesis have several processes in common [48] and genes involved in the mitochondrial iron metabolism are expressed in testis of both vertebrates and Drosophila. Therefore, it is not unlikely that our findings are applicable for vertebrates as well. Our study provides a first insight and tools in the form of characterized fly mutants, that will aid further investigations concerning the role of iron, and specifically mitochondrial iron metabolism during spermatogenesis.

Methods
Fly strains
w1118 and w1118; Vno/TM6c Sh, Tb flies were obtained from M.S. Dushay (Illinois Institute of Technology) and w1118; wg/Cyo; A2-3 Sb/TM6c, Tb flies and w1118, Sco/ SM1, Cy; Vno/TM3, Sb flies were obtained from P. Kylsten (Södertörns Högskola, Sweden). l(3)SH115 and Df(3R)ED6277 flies were obtained from Szeged Drosophila stock center, Hungary. Stocks of dmfrnEY01302 and mfrnBG00456 flies were obtained from the Bloomington Drosophila stock center, USA. w1118; Sco/SM1, Cy; Vno/ TM6c, Sb Tb flies were made by conventional fly genetics. We genotyped dmfrnSH115, (SH115(3)SH115) dmfrnEY01302 and mfrnBG00456 flies to confirm the stocks (see Additional file 1, additional methods for genomic DNA extraction and PCR protocol).

The ~11 kbp large deletion Df(3R)ED6277 at cytogenic map position 98B6 was generated by recombination as part of the DrosDel project [49,50]. It removed genes dmfrn and CG5514 completely and parts of the putative 5’ UTRs of Mes-4 and Gp93. Because Df(3R)ED6277 was uncharacterized and unverified, we performed PCR confirmation (see Additional file 1, additional Figure A1B and A1F) as proposed by Ryder et al. [50] and sequenced the products, confirming the integrity of the recombination product.

Flies were kept on standard potato sucrose medium in a 12 h/12 h light/dark cycle. Fly stocks were kept at 18°C. Experiments and crosses were carried out at room temperature (22-25°C).

For histostaining and other microscopic work, adult male flies were separated from female flies after eclosion and testes were removed from one to three days old flies in PBS buffer using sharp tweezers.
P-element reversion screen

P-element reversion [21] was carried out by crossing female w1118; dmfrnSH115 virgins with w1118; wg/CyO; Δ2-3 Sb/TM6c Tb male flies that carry the immobilized transposase source Δ2-3 [51]. F1 males of the genotype w1118; CyO/+; Δ2-3 Sb/dmfrnSH115 (mosaic expression of white) were crossed to virgin w1118; Vno/TM6c Sb, Tb female flies. Single white eyed male F2 of the genotype w1118; +/-; w1118; Vno were crossed to virgin w1118; Vno/TM6c Sb, Tb. Finally, F3 w1118; 7/TM6c Sb, Tb siblings were used to establish lines. These were then analyzed by PCR for the absence of P[lacW]SH115C[3]SH115.

Fertility assay

Single 3-4 days old male or female flies, collected from flies reared on indicated food sources, were mated with 2-3 virgin w1118 female or 2 male w1118 flies reared on normal food, respectively. After 5-7 days of mating, the fraction of fertile flies was determined by the presence of larvae.

Transgenic dmfrnvenus flies

The genomic region of dmfrn was subcloned in several steps to generate a genomic construct, tagged by a C-terminal fusion with the coding sequence of the fluorescent protein venus. For all PCR reactions, Phusion high fidelity polymerase (Finnzymes) was used with either w1118 genomic DNA, or plasmid pHWV (Carnegie Drosophila Gateway' Collection) as a template for dmfrn or venus respectively. Restriction digestions were carried out with enzymes from New England Biolabs. Arctic shrimp alkaline phosphatase and the Rapid DNA Ligation Kit were obtained from ROCHE Applied Science.

Using primers 5’ ACT AGT CTA GGA GCA GCA GGC CCA C 3’ (introducing SpeI and a stop codon in the first exon of Gp93) and 5’ AAA AAT CGA TAA AAG CTA GCC GTG CTG AAG CCC CGC TCG 3’ (introducing Nhel and Clal) the region from the first exon of Gp93 to the end of the coding sequence of dmfrn, omitting the stop codon, was subcloned into pCR-XL-TOPO (Invitrogen) by TOPO cloning (Invitrogen). Using primers 5’ AAA AGC TAG CAT GGT GAG CAA GGG CGA G 3’ (introducing a Nhel site) and 5’ AAA AAT CGA TTC ACG TGG ACC GGT GCT T 3’ (introducing a Clal site), the coding sequence of the fluorescent protein venus was PCR amplified, and cloned in frame behind the coding sequence of dmfrn using restriction sites Nhel and Clal.

Next, the 3’UTR of dmfrn was PCR amplified, using primers 5’ AAA AAT CGA TAC GTA GGC GTG GGC GTG GG 3’ (introducing Clal) and 5’ AAA AGG TAC CCG GAA ACA ATA AAA GGC AAT TGT TG 3’ (introducing a KpnI site) and was cloned behind the coding sequence of venus, using the introduced Clal site of the previous step and a KpnI site within the plasmid. Cloned fragments were sequenced after each step. Finally, the genomic construct was cloned into pCasper4 [52] using restriction sites SpeI and KpnI and the resulting plasmid was sent for co-injection with plasmid pΔ2-3 into w1118 embryos for generation of transgenic flies at the Department of Developmental Biology, Wenner-Gren Institute, Stockholm University. Transgenic dmfrnvenus flies were verified by PCR and outcrossed for four generations to w1118 flies.

RT-RT PCR

RNA was extracted from tissues prepared from 2 to 3 days old virgin male w1118 adult flies, raised at room temperature on potato food. Dissections were carried out in PBS buffer on a wax plate on ice and tissues were dissolved directly in 400 μL 1% 2-mercaptoethanol RLT buffer from the RNeasy Kit (QIAGEN). Heads and thoraxes were ripped open before lysis and disrupted using a micro pestle. Tissues from 20 flies were pooled per experiment and after passing lysates through QIAshredder columns (QIAGEN), RNA was purified from 350 μL flow through using the RNeasy Kit (QIAGEN). Integrity of RNA was analyzed spectrophotometrically and by agarose gel electrophoresis. cDNA was synthesized from 350 ng total RNA using the QuantiTect reverse transcription kit (QIAGEN) performing the gDNA wipeout reaction to remove genomic DNA contaminations. The cDNA synthesis reaction was also performed without QScript RTase and used as a negative control PCR reaction to test for gDNA contamination. The QuantiTect SYBR green kit (QIAGEN) was used for RT-RT PCR in a RotorGene 3000 (Corbett Research) thermocycler using primers for cDNAs of genes Rp49 [53], dmfrn, Fer1HCH, Fer2LCH [13], Fer3HCH (CG4349), forward 5’- GAA GGC ATC GCG ACC CAA CCC ong TAG CTA GCg 3’ and the resulting plasmid was sent for co-injection with plasmid pΔ2-3 into w1118 flies.

X-gal Staining

Testes were fixed in 3.7% (w/v) formaldehyde in PBS for 15-30 min at RT. Fixed testes were washed twice for 10 min with PBS, permeabilized for 20 min with PBST (PBS, 0.3% (v/v) Triton X-100) and then stained with staining solution (10% (w/v) 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) in dimethyl sulfoxide was added to a final concentration of 0.2% (w/v) to preheated staining buffer (10 mM phosphate buffer pH 7.2, 150 mM NaCl, 1 mM MgCl2, 3 mM K3[Fe(II)(CN)]6, K4[Fe(III)(CN)]6, 0.3% (v/v) Triton X-100) at 37°C until colorization was visible. Testes were first rinsed with NaCl-T (0.7% (w/v) NaCl, 0.3% (v/v) Triton X-100) then with water, mounted in PBS and imaged by conventional light microscopy.
Testes preparation, testes squashes and fluorescence staining of testes and fluorescence microscopy

Testes of 1-3 days old virgin males were dissected out in a drop of PBS buffer using fine forceps (#5, FineScienceTools), transferred to a small drop of PBS on a microscope slide and carefully ripped open to release their content under the weight of a coverslip. Excess buffer was removed using a paper cloth, while observing the squash under phase contrast settings on a Leica TSC-SP microscope.

For fluorescence microscopy, testes were collected in Schneider Drosophila Medium (SDM) and stained with 100 nM MitoTracker Deep Red 633 nm (Invitrogen) in SDM for 2 hours. Testes were washed twice with PBS, fixed with 3.7% (w/v) formaldehyde in PBS, washed twice with PBS and permeabilized with 0.3% (v/v) Triton X-100 in PBS. After washing twice with PBS, testes were stained with Rhodamine Phalloidin (diluted 1:1000 in PBS; Invitrogen), washed three times with PBS and mounted in VECTASHIELD Mounting Medium with or without DAPI (Vector Labs). Specimens were either imaged using conventional fluorescent microscopy on a Leica TCS-SP or confocal laser scanning microscopy on a Leica TCS-NT or a Zeiss LSM510 confocal microscope.

Transmission electron microscopy

Glutaraldehyde fixed testes were dehydrated, embedded, sectioned and stained following standard procedures at the Biological Structure Analysis Facility, Uppsala University, Sweden. Sections were imaged using a Zeiss Supra35VP electron microscope.

Additional material

Additional file 1 Confirmation of fly lines used in the study, spermatogenesis defect of dmfrmrnu_10935472; Df(3R)ED6277 flies and localization of ferritin during spermatogenesis. Contains text, a table with primers and images.

Authors’ contributions

CM designed and performed all experiments, interpreted the results, drafted and wrote the manuscript. MIL discussed the experiments and results and critically read the manuscript. Both authors read and approved the final manuscript.

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