Overexpression of Human and Fly Frataxins in Drosophila Provokes Deleterious Effects at Biochemical, Physiological and Developmental Levels

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Abstract

**Background:** Friedreich’s ataxia (FA), the most frequent form of inherited ataxias in the Caucasian population, is caused by a reduced expression of frataxin, a highly conserved protein. Model organisms have contributed greatly in the efforts to decipher the function of frataxin; however, the precise function of this protein remains elusive. Overexpression studies are a useful approach to investigate the mechanistic actions of frataxin; however, the existing literature reports contradictory results. To further investigate the effect of frataxin overexpression, we analyzed the consequences of overexpressing human (FXN) and fly (FH) frataxins in Drosophila.

**Methodology/Principal Findings:** We obtained transgenic flies that overexpressed human or fly frataxins in a general pattern and in different tissues using the UAS-GAL4 system. For both frataxins, we observed deleterious effects at the biochemical, histological and behavioral levels. Oxidative stress is a relevant factor in the frataxin overexpression phenotypes. Systemic frataxin overexpression reduces Drosophila viability and impairs the normal embryonic development of muscle and the peripheral nervous system. A reduction in the level of aconitase activity and a decrease in the level of NDUF3 were also observed in the transgenic flies that overexpressed frataxin. Frataxin overexpression in the nervous system reduces life span, impairs locomotor ability and causes brain degeneration. Frataxin aggregation and a misfolding of this protein have been shown not to be the mechanism that is responsible for the phenotypes that have been observed. Nevertheless, the expression of human frataxin rescues the aconitase activity in the fh knockdown mutant.

**Conclusion/Significance:** Our results provide in vivo evidence of a functional equivalence for human and fly frataxins and indicate that the control of frataxin expression is important for treatments that aim to increase frataxin levels.

Introduction

Friedreich’s ataxia (FA), an autosomal recessive disease, is the most frequent form of inherited ataxias in the Caucasian population (1:50000) [1]. The major cause of this disease is the presence of a large GAA repeat expansion in the first intron of the FXN gene [2]. This large GAA repeat decreases the level of transcription of the mRNA that encodes the protein frataxin [3,4], resulting in levels that range from 5% to 30% of the normal level of this protein [5]. The clinical manifestations of FA involve spinal cord and cerebellum neurodegeneration, which cause gait and limb ataxia, muscular weakness and speech impairments [6,7]. Other manifestations of FA include scoliosis, diabetes and hypertrophic cardiomyopathy, which is the main cause of death [8].

Frataxin is a highly conserved protein throughout evolution [9]. This degree of conservation has enabled the development of FA models in many organisms, from E.coli to the mouse, that have contributed to a better understanding of this protein’s function; however, the exact function of frataxin remains elusive. Seminal findings by a number of key studies have suggested potential roles for frataxin in iron homeostasis [10–14], as an activator of the respiratory chain [15–17], as a regulator of Fe-S cluster assembly through activation [12,18–23] or inhibition [24] and/or by promoting cellular defense against reactive oxygen species [25–32].

In Drosophila, the frataxin homolog (fh) shares a high degree of sequence conservation and projected folding with other frataxin orthologs [33]. Moreover, a reduction in the level of frataxin expression in Drosophila has been established as an effective model to study frataxin function and the pathological mechanisms that underlie frataxin deficiency. In fact, the loss of fh recapitulates important behavioral and biochemical features of human disease...
Furthermore, Drosophila models have provided support for the crucial involvement of oxidative stress, particularly peroxides, in the development of FA [31,32,33]. These models have also indicated that frataxin is relevant in glial cells and that these cells play a role in FA [35]. In addition, these models have revealed that mitochondrial depolarization is an initial element in the axonal transport defects that lead to a concomitant dying-back neuropathy [30]. Overexpression studies in the fruit fly have been greatly used to study the gene’s function and to provide insight into the human inherited pathologies. Among these studies, several reports of investigations of the ectopic expression of human genes in Drosophila have provided highly valuable information regarding Alzheimer’s disease [37], polyglutamine diseases [38], Parkinson’s disease [39,40] and dominant spinocerebellar ataxias (SCAs) [41,42].

For frataxin overexpression, the existing literature presents contradictory results. Experiments in mice [17,43] or in cultured cells [15,44,45] have revealed that frataxin overexpression was innocuous or had a positive effect on the cell’s biology, simulating the production of ATP or inducing the recruitment of antioxidant defenses. Similarly, Runko et al. [46] reported that the overexpression of Drosophila frataxin promoted cellular resistance to oxidative stress. However, we have previously reported that Drosophila frataxin overexpression [31] leads to detrimental phenotypes in the fly, including developmental defects, a decrease in the level of aconitate activity and hypersensitivity to oxidative stress. Notably, the overexpression of frataxin in yeast has also been shown to critically affect aconitate activity [47].

In the present study, we analyzed the effects of the overexpression of two frataxins in a multicellular organism, Drosophila melanogaster. To achieve this aim, we generated transgenic flies that overexpressed human (FH) frataxins through the UAS/GAL4 system. We also studied whether FXN can functionally replace endogenous Drosophila frataxin. In the present paper, we report that the increased expression of human or fly frataxin in Drosophila leads to deleterious effects at biochemical, histological and behavioral levels. We also show that FXN can rescue the reduction in the aconitate activity that is associated with the loss of frataxin in the fly. Our results provide in vivo evidence of a functional equivalence between human and fly frataxins and indicate that the regulation of frataxin expression is a key factor that underlies frataxin function.

Materials and Methods

Drosophila stocks

The w¹¹¹⁸ strain of Drosophila was used as the control line and for the injection of the UAS-FXN construct. The UAS-FH line, which carried the FH coding sequence under the control of UAS, was previously generated in our laboratory [31]. The UAS-FH line induced a 9-fold increase in the level of FH-mRNA at 29°C. The UDH2 line was kindly provided by J.P. Phillips (University of Guelph, Guelph, ON). UDH2 induced a strong interference of FH, and the FH protein was reduced to undetectable levels [34]. The MitoCat flies were a gift from W. Orr (Southern Methodist University, Dallas, USA). The actin-GALA, da-GAL4, 24B-GALA, new-GAL4, repo-GALA and Appl-GALA driver lines were obtained from the Bloomington Stock Center. The stocks were maintained at 25°C using standard cornmeal agar medium. The crosses between the GAL4 drivers and the UAS responder lines were conducted at either 25°C or 29°C. The rescue experiments were conducted by generating the following stocks: mitoCat/CyO; UAS-FXN/TM3; and UDH2/CyO; UAS-FXN/TM3.

Construction of the UAS-FXN transgene and the generation of the fly transmorphants

The cDNA for FXN was obtained from human fetal brain poly(A)+ mRNA (Invitrogen). A 645 bp fragment, which included the entire coding region of the gene, was amplified using the following primers: FXN-pUAST1 (CTCGAGATGGACTCTGGCGG-GCCG) and FXN-pUASTr (GGTACCTCAAGGATCTTTT-CCGGGATAGGCGCAA). This fragment was inserted into the pCR2.1-TOPO vector and then subcloned into the pUAST vector to generate the UAS-FXN transgene.

The transgenic flies were generated using standard embryonic injection protocols [48]. Seven independent lines were obtained, and the presence of the transgene was verified in each line using PCR with vector-specific primers. The sequencing of the PCR products revealed that there were no mutations present in the FXN sequence. The lines were examined for the expression of FXN by crossing each of these lines with the actin-GAL4 driver line. A line that contained the UAS-FXN transgene on the second chromosome was selected to perform our experiments.

Western blotting

The total protein extraction from the Drosophila larvae was performed as previously described [49]. The protein levels were determined using the Bradford assay. The samples were separated on 5% stacking, 15% separating SDS polyacrylamide gels. The resolved proteins were electroblotted to a Hybond-ECL nitrocellulose membrane (GE Healthcare) and were probed using mouse anti-FXN (Chemicon, Millipore, 1:2000) or mouse anti-NDUFS3 (Mitosciences MS112, 1:2000) antibodies. Fluorescent goat anti-mouse was used as the secondary antibody in these cases. Detection and quantification was conducted using the Odyssey system (Li-cor Inc.). Alternatively, goat anti-mouse IgG horseradish peroxidase conjugate (Sigma-Aldrich) was used as a secondary antibody and was detected using ECL Detection Reagent (GE Healthcare).

Immunohistochemistry staining

The whole mount embryo staining technique with horseradish peroxidase was conducted as previously described [50]. The embryos were incubated with the following primary antibodies: mouse mAb anti-myosin heavy chain (anti-MHC), 1:8 dilution, a gift from D. Kiehart; mouse mAb 22C10 anti-peripheral nerve system neurons (anti-PNS), 1:50 dilution; mouse mAb BP102 anti-central nerve system axons (anti-CNS), 1:200 dilution, from the Developmental Studies Hybridoma Bank; and rabbit anti-even-skipped protein, 1:2000 dilution, kindly provided by M. Frasch. The Ab-antigen complexes were detected using biotinylated horse anti-mouse IgG (Fierce, Rockford, IL) or biotinylated goat anti-rabbit IgG (Pierce) antibodies.

Brain histology

For the examination of the adult fly brains using light and electron microscopy, ultrathin Epon plastic sections were post-stained with 2% uranyl acetate, which was followed by Reynolds’ lead citrate. Next, the sections were stabilized for transmission electron microscopy using carbon coating. The examination was conducted using a Zeiss EM10C/VR electron microscope at 80 kV. The glial cell material was identified by its characteristic higher electron density.

Life span determination and climbing assay

For the life span determination, the male flies were collected within 24 h of eclosion and were raised at 25°C under a 12 h:12 h
light/dark cycle. These flies were transferred to fresh food vials every 2–3 days. The climbing assay was conducted as described in Botella et al. [51].

Assay of the aconitase activity
The total aconitase activity was determined from L3 larvae using the Bioxytech Aconitase-340™ Spectrophotometric Assay Kit (Oxis International Inc, Portland, OR).

Hyperoxia treatment
The hyperoxia treatment was started one day post-eclosion and was performed as previously described [31]. To measure the aconitase activity, L3 larvae were maintained in hyperoxia conditions for a 24 h period before performing the assay.

Gel filtration chromatography
The mitochondria from the actin-GAL4>UAS-FXN larvae were isolated (MITOISO1, Sigma), lysed in hypotonic buffer (HEPES 10 mM, pH 7.0) and sonicated (three times for 30 sec) before being centrifuged at 20,000 g for 30 min. The mitochondrial matrix proteins were subjected to size exclusion chromatography on a Superdex 200 10/300 GL column with a fractionation range of 10 to 600 kDa (GE Healthcare) and were eluted with 50 mM HEPES and 140 mM NaCl, pH 8.0, at a flow rate of 0.5 ml/min. Blue Dextran 2000 (1 mg/ml) was used to estimate the void volumes, and gel filtration molecular weight standards (GE Healthcare) were used to calibrate the column. An equal volume of each fraction was analyzed using SDS-PAGE and western blotting.

Statistical analysis
A Kaplan-Meier analysis of the survival data with a semi-parametric log rank test was performed using Graph Pad Prism 4.0 software. The differences in the locomotor and aconitase activities were tested using a one-way ANOVA test, using the Statistical Packages for the Social Sciences (SPSS) v17.0. A value of p<0.05 was considered to be statistically significant.

Results
Human frataxin is correctly expressed and targeted to the mitochondria in Drosophila
To investigate the effect of human FXN expression in D. melanogaster, we generated transgenic flies that carried the UAS-FXN construct. These flies were crossed with the da-GAL4 driver line at 25°C to reach the ubiquitous expression of the human gene. Because the da-GAL4>UAS-FXN individuals exhibited lethality before adult eclosion, the presence of human frataxin was confirmed in the transgenic larvae using western blotting. As expected, human frataxin was only detected in the da-GAL4>UAS-FXN larvae; no signal was observed in the driver and responder controls (Figure 1A). To test whether FXN was transported into the mitochondria, we analyzed the relative amount of frataxin in the mitochondrial and cytosolic fractions. We used an anti-actin antibody as a control for cytosolic contamination. Anti-actin staining revealed abnormalities in the junctions of lateral transversal muscles 1, 2 and 3 and the ventral longitudinal muscle 1 (C) compared with the control (B). Moreover, a few embryos exhibited abnormalities in the axonal path finding of the sensory nerves (G) with respect to the control (F).

Figure 1. Effect of FXN overexpression in the embryonic development. (A) Detection of the FXN protein in da-GAL4>UAS-FXN (+,+ ) larvae in the mitochondrial (mito) and the cytosolic (cyto) fractions. The control genotypes of the larvae were da-GAL4>yw (+,−) and yw>UAS-FXN (−,−). The human frataxin protein was localized in the mitochondria. Anti-actin was used as a control for cytosolic contamination. (B–G) Muscular and nervous system defects in da-GAL4>UAS-FXN and actin-GAL4>UAS-FXN embryos at stage 16. In these panels, anterior is toward the left, and all of the views are lateral views. Anti-myosin staining revealed abnormalities in the junctions of lateral transversal muscles 1, 2 and 3 and the ventral longitudinal muscle 1 (C) compared with the control (B). Moreover, a few embryos exhibited abnormalities in the axonal development of mutant (E) versus control (D) embryos. Staining with 22C10 detected strong abnormalities in the junctional F actin of the sensory nerves (G) with respect to the control (F).

doi:10.1371/journal.pone.0021017.g001

Human frataxin overexpression reduces Drosophila viability
We have previously reported the consequences of increasing the amount of fh expression in Drosophila [31]. We found that the general and mesodermal overexpression of fh at 29°C resulted in lethality during the pre-adult stages and restricted the expression of fh in the nervous system, which had no effect on viability.

To test whether the effect of FXN overexpression on Drosophila viability was similar to the effect of the overexpression of fh, we first investigated systemic FXN expression with the ubiquitous da-GAL4 and actin-GAL4 drivers by mating the flies at 25°C and 29°C. Full lethality was observed with both drivers at both temperatures. These results were similar to those after the fh overexpression (Table 1); however, deaths were observed earlier in the individuals with FXN expression than in those with fh overexpression.

Next, we examined the consequences of a tissue-specific expression of FXN. The FXN expression was specifically driven
in the nervous system and in the muscles, which are two of the most affected tissues in patients with FA. The FXN expression in the embryonic mesoderm (24B-GAL4) led to the death of all of the individuals that were early to late pupae. However, we observed viable progeny that expressed FXN in the central nervous system (CNS), in the sensory organs and their precursors and in the glial cells, using the Appl-GALA, neu-GALA and repo-GALA drivers, respectively. In contrast, the expression of FXN at 29°C with the neuronal post-mitotic driver Appl-GAL4 resulted in pre-adult lethality (Table 1).

Collectively, these results demonstrate that tissues respond similarly to Drosophila and human frataxin overexpression and that an adequate balance of the systemic synthesis of frataxin is critical for fly viability.

### The general expression of FXN disrupts normal Drosophila development

Immunohistochemical staining was conducted in da-GAL4-UAS-FXN and actin-GALA-UAS-FXN embryos to identify the underlying defects that were associated with the lethal phenotypes. Anti-myosin staining of the muscular system revealed defects in the junctions of lateral transversal muscles 1, 2 and 3 with ventral longitudinal muscle 1, which was likely due to deficient muscle growth (Figure 1C). Defects in several muscles were also reported after fh overexpression [31]. In a few cases, we observed FXN-expressing embryos that exhibited a disrupted muscular system (Figure 1E). After the embryo’s PNSs were stained using the 22C10 antibody, a strong disorganization of the sensory axons was detected (Figure 1G). Similar alterations were described for the da-GAL4 driven overexpression of fh [31]. No abnormalities were found in the CNS of the FXN-expressing embryos when they were stained using the BP102 antibody (as in da-GAL4-UAS-fh embryos; data not shown).

We observed that the lethal phenotypes that were associated with the general overexpression of human or fly frataxin mainly resulted from the impairment of correct muscle and PNS development, whereas the CNS was not affected. The high degree of similarity between the defects that were observed with the overexpression of human and fly frataxins supports the involvement of the overexpression of FXN in the same developmental mechanisms than FH overexpression. In addition, these results indicate that the frataxin level is critical for the normal embryonic development of muscle and the PNS.

### Nervous system expression of FXN shortens life span, impairs locomotor performance and causes brain degeneration

We further assessed whether human frataxin expression in neural tissues affects Drosophila fitness during adulthood. The length of the life span was examined using the Appl-GAL4, neu-GAL4 and repo-GAL4 drivers. The FXN flies exhibited a statistically significant decline in the mean (75%, 80% and 50%, respectively) and maximum life spans (74%, 75% and 56%, respectively). These decreases were larger than those that were observed after the overexpression of endogenous fly frataxin (Figure 2A–C).

To study the effect of frataxin overexpression on the nervous system functioning in Drosophila, the locomotor activity of the flies was analyzed. The overexpression of human or fly frataxin reduced the climbing ability of the flies in an age-dependent manner for all three of the drivers used. The larger reduction was observed for FXN with neu-GAL4 driver, which exhibited a 70% reduction in the 5-day-old flies and a 90% reduction in the 10-day-old flies (Figure 2E). Appl (Figure 2D) and repo-GAL4 (Figure 2F) also induced locomotor dysfunctions, although to a lesser extent (55% and 25% in 5-day-old flies, respectively). The findings indicate that aging appears to exacerbate the reduction in locomotor ability that results from frataxin overexpression.

To identify the cellular pathology underlying the life span and locomotor phenotypes that have been associated with frataxin overexpression, the brain sections from flies that overexpressed human frataxin were analyzed using light and electron microscopy. The selective FXN expression in glial cells induced a strong age-related degeneration in the cortex and a neuropil vacuolization with the presence of droplet-like structures (Figure 3D). An ultrastructural analysis revealed a complete morphological disruption of the glial cells and the concomitant formation of lipid droplets (Figure 3E). Notably, a very similar phenotype was observed in the glial cells that lacked Drosophila frataxin [35]. Moreover, as shown in Figure 3F, several regions of the brain exhibited clear mitochondrial phenotypes, such as an abnormal morphology or vacuolization. Although the Appl-GAL4 > UAS-FXN flies exhibited a clear locomotor deficit and a shortened life span, these flies did not display brain abnormalities compared to the control age-matched individuals (data not shown).

These results indicate that an excess of frataxin impairs embryonic development and negatively affects fly fitness. Remarkably, our results from glial cells may suggest that frataxin overexpression alters cellular homeostasis in a similar manner to frataxin knock-down. These data indicate that a balance of frataxin levels is critical for the correct functioning of several cell types in the Drosophila nervous system.

### Overexpression of human frataxin enhances susceptibility to oxidative stress

One of the most characteristic biochemical defects that is associated with a loss of frataxin is the reduction of aconitase activity [18,19,31]. Therefore, we tested whether the overexpression of human frataxin in Drosophila would also affect the activity of this enzyme. Aconitase activity was measured in actin-GAL4-UAS-FXN L3 larvae because the ubiquitous expression of FXV caused lethality before adult eclosion (Table 1). Notably, these larvae exhibited a 50% reduction in aconitase activity under normoxia conditions (Figure 4A).

Several FA models have also resulted in a strong reduction of other Fe-S-cluster-containing proteins, such as the complex I subunit, SdhA, SdhB or the Rieske protein [32,33]. Thus, we...
examined whether frataxin overexpression would produce a similar effect on the NDUF53 levels. In agreement with the results of other studies [52,53], we observed that FXN overexpression triggered a 40% reduction in the amount of NDUF53 protein expression (Figure 4B) without a change in the mRNA levels (data not shown), which excludes the possibility of a transcriptional regulatory mechanism. The reduction of the aconitase activity and the decrease in the amount of NDUF53 expression are direct evidence of a reduction in the Fe-S cluster formation in our overexpression model.

Given that aconitase is a specific target of oxidative stress [54,55], we assessed the functional integrity of aconitase in FXN larvae after oxidative stress injury. As expected, the ubiquitous expression of FXN, combined with a hyperoxia treatment, also resulted in a two-fold reduction in aconitase activity compared to hyperoxia-treated controls (Figure 4A). Moreover, aconitase was seriously affected in flies with general fh overexpression that was combined with hyperoxia [31].

To test whether oxidative stress was involved in the phenotypes that were observed in the nervous system, we exposed flies that overexpressed FXN to a highly oxidative atmosphere (99.5% O2). Under these conditions, we observed a strong decrease in the mean (65%) and maximum life span (50%) when compared to those of the controls (Figure 4C–E). Again, the FXN flies displayed stronger phenotypes than the flies that overexpressed fh.

In *Drosophila*, a constitutive increase in the mitochondrial-driven catalase (*mitoCat*) activity is known to improve the resistance to oxidative damage [56]. Moreover, the expression of this enzyme has been reported to extend the life span of frataxin-deficient flies and to improve the resistance of these flies to oxidative insult [32]. Therefore, we examined the effect of this free radical scavenger on life span and locomotor performance in our frataxin overexpressing flies. As illustrated in Figure 4F, *mitoCat* produced a significant prolongation of life span when frataxin was overexpressed using *neur-GAL4* and caused a statistically non-significant increase for repo-GAL4 (Figure 4G). In addition, *mitoCat* ameliorated the climbing deficiency that was induced by FXN or fh overexpression in glial cells (Figure 4H).

These results clearly identify oxidative stress and mainly hydrogen peroxides as key factors in the frataxin overexpression phenotypes that have been observed.

**Overexpressed FXN do not form aggregates or misfold in *Drosophila***

The overexpression of human or *Drosophila* frataxins produce a phenotype that is surprisingly similar to the phenotype that is observed in frataxin-depleted mutants. Thus, we assessed whether the overexpression of frataxin would induce neomorph phenotypes and lead to a loss of function phenocopy via protein aggregation or misfolding.

Heat shock proteins have been reported to display rescuing effects in *Drosophila* neurodegenerative models of protein misfolding or aggregation [57–59]. Therefore, we assessed whether the co-expression of heat-shock proteins would lead to beneficial effects in our frataxin overload scenarios. Human heat-shock cognates were used in combination with FXN, and *Drosophila* Hsp70 and Hsp22 were co-expressed with fh; however, these heat-shock proteins were not able to improve the climbing performance of the frataxin-overexpressing flies (data not shown).

Size exclusion chromatography was conducted for the mitochondrial matrix proteins from the FXN-overexpressing larvae, and the fractions that were obtained were analyzed using western blotting for the presence of FXN. In our experiments, after the overexpression of FXN in *Drosophila*, human frataxin was recovered.
as a monomeric form (Figure 5A), and no high molecular weight frataxin aggregates (dimers, trimers or multimeric forms of frataxin) were detected in the void volume (fraction 14). It was nevertheless possible that frataxin protein aggregates were not solubilized with the mitochondrial matrix proteins. Insoluble cellular proteins were solubilized, and the western blot did not reveal the presence of frataxin in this solubilized fraction (data not shown).

Collectively, these results indicate that frataxin aggregation or misfolding is unlikely to be the mechanism behind the phenotypes that have been observed.

**FXN overexpression restores the aconitase activity in fh deficient individuals**

In terms of their sequences and other structural properties, the degree of conservation between FH and FXN [33,60] indicates a possible similarity in the function of these proteins. In support of this hypothesis, we have shown that Drosophila and human frataxin overexpression produce similar phenotypes. Lastly, we investigated the consequences of expressing FXN in fh-knockdown flies. To accomplish this task, we generated flies by combining FXN and UDIR2 transgenes, and the latter transgene induced a 90% reduction in the level of fh expression [34].

A decrease in the level of aconitase activity appears to be the most sensitive biological and biochemical marker in the FA fly models [31–35]. As a result, we tested whether aconitase could be rescued in fh knockdown flies expressing FXN. In agreement with previously published results [34], the depletion of fly frataxin led to a two-fold reduction in aconitase activity in larvae compared to controls. Remarkably, the expression of FXN prevented aconitase inactivation, and the aconitase activity was recovered to levels that were comparable with those of the controls (Figure 4A).

Considering the possibility that the rescue of the aconitase activity was related to degradation of the FXN mRNA that was induced by the RNAi transgene directed to fh, we assessed the human frataxin protein levels using western blotting. Comparable levels of FXN were observed in the actin-GAL4/UAS-FXN and actin-GAL4>UAS-UDIR2/UAS-FXN larvae (Figure 4B).

We can conclude that human frataxin is able to replace the endogenous Drosophila frataxin, which suggests that these proteins play an equivalent role in the cell biology of these organisms.

**Discussion**

Friedreich’s ataxia is the most common autosomal recessive ataxia in the Caucasian population. This disease exhibits an irreversible progression that confines a patient to a wheelchair and leads to an early death. Moreover, although different treatments are currently being developed and assessed in clinical trials, there is no cure available. To generate effective and adequate therapies for Friedreich’s ataxia, it is imperative to define the function of the frataxin protein. Unfortunately, the precise function of this protein is still a matter of debate. Although overexpression studies do not represent a disease model for FA, these studies are a useful approach to decipher the mechanism of action of frataxin.
Furthermore, these models may provide insight into the effects of an excess of frataxin, which is a critical factor for the validation of treatments that are based on an increase in this protein’s expression.

Frataxins are a highly conserved family of proteins. In silico analyses have shown that *Drosophila* frataxin and frataxin proteins that are found in other species share large percentages of identity and similarity in their sequence and a common secondary structure [33]. The closest match between the human and the fly frataxin involves a stretch of 38 amino acids at the C-terminus, which is encoded by *fh* exon 2, and exons 4 and 5a of the *FXN* gene, respectively. This highly conserved region is very likely to form a functional domain with a β-sheet structure that is flanked by α-helices, where the sequence is less conserved [33]. Moreover, *Drosophila* frataxin has similar biophysical properties to human frataxin [60] and exhibits a mitochondrial localization [31]. In agreement with mouse models of FA [19], a strong systemic depletion of *fh* induces lethality during early development [31,34], whereas the moderate reduction of *fh* produces phenotypes that parallel the symptoms of FA patients [31]. In addition, the tissue-specific silencing of frataxin leads to the mimicking of human phenotypes [31,34–36]. Collectively, these data indicate that these proteins may be playing identical roles; however, their functional equivalence had not yet been demonstrated experimentally.

In the present work, we generated a *Drosophila* strain that overexpressed the human frataxin (*FXN*). Flies with an increased

![Figure 4. Molecular effects of frataxin overexpression and the involvement of oxidative stress.](image-url)

**Figure 4.** Molecular effects of frataxin overexpression and the involvement of oxidative stress. (A) The negative effects of human-frataxin overexpression on aconitase activity under normoxia and hyperoxia (99.5% O2) conditions. (B) Human frataxin overexpression triggered a reduction in the synthesis of the complex I subunit (amount normalized to the internal control α-tubulin). (C–E) Increased susceptibility to hyperoxia-mediated oxidative damage in flies overexpressing human and fly frataxin in the nervous system. (F,G) Constitutive expression of mitochondrial catalase (*mitoCat*) led to an extension of the mean and maximum life span of the flies with increased frataxin expression. This effect was strong in the peripheral nervous system (*neur-GAL4*) and moderate in the glial cells (*repo-GAL4*). (H) Co-expression of mitochondrial catalase (*mitoCat*) rescues (5d) and alleviates (10d) the locomotor deficits in the flies with an increased level of frataxin expression in the glial cells. The survival curves were analyzed using the Kapplan-Meier test. The level of significance in A, B and H was determined using a one-way ANOVA with a post hoc Newman-Keuls test (*p<0.05). The error bars represent the standard error.

doi:10.1371/journal.pone.0021017.g004
level of expression of FXN exhibited similar defects to those found after endogenous frataxin overexpression, such as an alteration of development, a reduction in viability, life span and motor ability [31] and clear manifestations of nervous system degeneration, impaired Fe-S cluster formation and an enhanced susceptibility to oxidative stress. These results support the hypothesis that these frataxins are functionally equivalent. In the present work, we have shown that FXN was able to recover aconitase activity, the most sensitive biochemical marker of FA, in frataxin-deficient larvae.

Although the effect of frataxin overproduction has been investigated in several models, our studies using Drosophila as a model are the only studies to show deleterious defects. Human frataxin overexpression has been reported to be innocuous in mice [43] or to stimulate energy production [17]. Frataxin promotes OXPHOS activation in cell culture [15,45] and increases cellular antioxidant defense in cell culture and yeast [44,47]. In agreement with these findings, the overexpression of frataxin in Drosophila that was conducted by Runko et al. [46] increased the resistance to oxidative stress and extended the life span of the flies. These differences may be related to the characteristics of the models but may also result from quantitative differences in the level of frataxin overproduction. A closer look at the data from these studies reveals that a slight overexpression of frataxin was reported, from 2 to 6 times that of baseline, depending on the model and/or the tissue that was used. Conversely, in the present work, and in Llorens et al. [31], a minimum of 9-fold increase in frataxin production was induced. Therefore, it is possible that moderate overproduction of this protein may lead to beneficial effects, whereas the expression of frataxin beyond a given threshold may have multiple effects inducing toxicity. Taken together, these results indicate that frataxin requires an optimal balance of its expression level to carry out its function properly. To date, very little is known about the regulation of frataxin expression. It has been reported that the transcription factors Hypoxia-inducible Factor 2alpha [61], SRF and TFAP2 [62] are involved in regulating the expression of frataxin, as may be the iron content of the cell [63]. Analyzing the mechanism of this regulatory network is a new field that will provide new targets for future therapies.

In agreement with the results of the present study, the overproduction of yeast frataxin has been shown to impair Fe-S cluster formation and to lead to a reduction in aconitase and SDH activities [47]. These authors proposed that the trimeric form of frataxin may be responsible for the interaction with the complex of iron-sulfur cluster machinery. Thus, the increase in frataxin oligomerization due to its overexpression would lower the amounts of trimers that restrict the production of the cluster. To clarify the toxic mechanism that is responsible for frataxin overexpression in Drosophila, we considered the possibility that FXN was also inducing protein aggregation or misfolding, leading to a reduction in the level of functional frataxin. However, the results of our gel filtration assays did not show any shift in FXN to higher molecular masses, and the protein was recovered in the monomeric form. In addition, the overexpression of human or fly heat-shock proteins did not lead to any improvement in the frataxin overexpression phenotypes. These results indicate that protein aggregation and misfolding are not the central factors leading to the frataxin overexpression defects. Notably, our results do not reproduce the data from the experiments using bacterial [64,65] or yeast [47,66] models in which frataxin forms multimers. These findings indicate that oligomerization does not occur in Drosophila. Our results are in agreement with the findings of Kondapalli et al. [60], who reported that FH seems to be less prone to aggregation in vitro than the yeast protein, which appeared as a monomer in most of the conditions that were tested.

In our overexpression model, we observedaconitase inactivation and a reduction in the NDUF3 levels, indicating an alteration in Fe-S cluster formation. These results argue in favor of the role of frataxin as an inhibitor of the Fe-S cluster assembly machinery, as previously suggested by Adinolfi et al. [24]. However, this inhibitory function is not consistent with the
stimulation of the respiratory chain that has been described by other authors [15,17,45]. Therefore, we propose that a moderate overexpression of frataxin may promote the synthesis of clusters or promote their stabilization when they are incorporated into apoproteins, as has been previously suggested [16,28]. In contrast, a larger overexpression of frataxin may lead to a reduction in Fe-S cluster formation, regardless of this protein’s function as an activator [12,18-23] or suppressor [24], by saturating the ISC machinery or by sequestering the proteins from the machinery that interact with frataxin. Frataxin has been recently proposed to maintain the ISCU/NFS1/ISD11 interaction [23]; however, an increase of frataxin may over-stabilize this complex and cause an Fe-S cluster deficiency.

Our results show that the constitutive expression of mitochondrial catalase demonstrate that oxidative stress and hydrogen peroxides are key factors in the frataxin overexpression phenotypes. Notably, the positive effect of mitochondrial catalase in counteracting frataxin defects has been previously reported [32]. These results suggest a common mechanism in the loss-of-function and gain-of-function phenotypes that are induced by frataxin. Remarkably, in Llorens et al. [31] and in the present study, other similar phenotypes between strong frataxin overproduction and frataxin depletion have been described, such as structural defects that lead to a reduction in longevity and locomotor capabilities and an increased sensitivity to oxidative damage. Moreover, the overexpression of FXN in glial cells leads to the presence of the lipid droplets and the brain degeneration that have been exhibited by glial-frataxin deficient flies [35].

References


In conclusion, we demonstrate that overexpression of Drosophila and human frataxins induces severe developmental problems in flies, a shortening of life span, brain degeneration and reduced aconitate activity. Moreover, the control of frataxin expression emerges as a crucial element for present and future treatments, such as gene therapy approaches, aimed at increasing frataxin levels.

Acknowledgments

The authors thank Dr. Dan Kiehart and Dr. Francesca Palau for the generous gifts of the anti-MHC and anti-Frataxin, respectively. The hybridrobioida antibodies 22C10 and BP102, which were developed by S. Benzer and C. Goodman, respectively, were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by the University of Iowa, Department of Biological Sciences, IA 52242. We also thank the Bloomington Stock Center for the fly stocks. The authors would like to thank Uschi Roth for their technical assistance, and Dr. Juan Ferré and Dr. Emanuel Lesuisse for their technical assistance and protocol for the gel filtration experiment. We also thank the Servicio Central de Soporte a la Investigación Experimental de la Universitat de València for access to the DNA analysis resources and databases.

Author Contributions

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