Altered lipid metabolism in a *Drosophila* model of Friedreich's ataxia

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Friedreich's ataxia (FRDA) is the most common form of autosomal recessive ataxia caused by a deficit in the mitochondrial protein frataxin. Although demyelination is a common symptom in FRDA patients, no multicellular model has yet been developed to study the involvement of glial cells in FRDA. Using the recently established RNAi lines for targeted suppression of frataxin in *Drosophila*, we were able to study the effects of general versus glial-specific frataxin downregulation. In particular, we wanted to study the interplay between lowered frataxin content, lipid accumulation and peroxidation and the consequences of these effects on the sensitivity to oxidative stress and fly fitness. Interestingly, ubiquitous frataxin reduction leads to an increase in fatty acids catalyzing an enhancement of lipid peroxidation levels, elevating the intracellular toxic potential. Specific loss of frataxin in glial cells triggers a similar phenotype which can be visualized by accumulating lipid droplets in glial cells. This phenotype is associated with a reduced lifespan, an increased sensitivity to oxidative insult, neurodegenerative effects and a serious impairment of locomotor activity. These symptoms fit very well with our observation of an increase in intracellular toxicity by lipid peroxides. Interestingly, co-expression of a *Drosophila* apolipoprotein D ortholog (*glial lazarillo*) has a strong protective effect in our frataxin models, mainly by controlling the level of lipid peroxidation. Our results clearly support a strong involvement of glial cells and lipid peroxidation in the generation of FRDA-like symptoms.

INTRODUCTION

Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disease affecting the central (CNS) and peripheral (PNS) nervous systems (1,2). The FRDA neuropathology typically presents early degeneration of large sensory neurons in the dorsal root ganglia (DRG), followed by degeneration of sensory posterior columns, spinal-cerebellar tracts and cortical-spinal motor tracts together with atrophy of the large sensory fibers in peripheral nerves (reviewed in 3) and grumose degeneration in the dentate nucleus (4). FRDA is also characterized by hypertrophic cardiomyopathy that becomes the main cause of death among the patients (5) and by accumulation of labile iron in patient's mitochondria (6).

FRDA is caused by a decreased expression of the mitochondrial protein frataxin (7,8). Frataxin deficiency leads to a dysfunction of the respiratory chain complexes and Krebs cycle components mainly due to an inappropiate Fe−S cluster synthesis, therefore provoking a bioenergetic failure and the subsequent cell death (9–11).

Frataxin-deficient cells are hypersensitive to oxidative insult pointing to oxidative stress as a key factor in FRDA pathology (12–17). Furthermore, samples from FRDA patients normally show increased levels of oxidative stress markers such as malondialdehyde (MDA, a lipid peroxidation product), 8-hydroxy-2′-deoxyguanosine (an indicator of oxidized DNA) and higher glutathione transferase activity (18–20, respectively).

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Dysregulated lipid metabolism and increased lipid peroxide levels are recurrent events in pathologies related to mitochondrial dysfunction (21–24). In addition, lipid imbalance has been reported to have a pivotal effect in the pathophysiology of multiple neurological disorders and neurodegenerative diseases, including bipolar disorders, schizophrenia, Alzheimer's, Parkinson's, Niemann–Pick and Huntington's diseases (reviewed in 25,26). Furthermore, as recently reviewed by Lessing and Bonini (27), *Drosophila melanogaster* has provided an impressive variety of examples which link mutations in lipid metabolism genes with neurodegenerative phenotypes such as *bgm* [*bubblegum*, encoding a fatty acid (FA) coenzyme A ligase], *eas* (ethanolamine kinase) or *SNF4Aγ* (encoding a subunit of AMP-activated protein kinase) or *fumble* (pantothenate kinase). Interestingly, iron metabolism is also disturbed in pantothenate kinase-associated neurodegeneration patients.

Very little is known about lipid homeostasis in FRDA. Although a typical symptom of FRDA is diabetes mellitus, several studies from patient autopsies or plasma samples showed undetectable changes in total cholesterol, triacylglycerides (TAG) and FA profiles compared with age-matched controls (28–30). However, levels of phosphatidylethanolamine, phosphatidylserine and linoleic acid were decreased (31,32). Nevertheless, electron microscopy analysis of the neuron/cardiac muscle frataxin-deficient mouse line revealed the presence of numerous lipid droplets in cardiac muscles (33).

Glial lazarillo (*GLaz*) is one of the *Drosophila* homologs to apolipoprotein D (ApoD), a human protein whose expression is increased in different neurological disorders such as Alzheimer's disease, schizophrenia and in the aging brain (reviewed in 34). Both, vertebrate ApoD and *Drosophila GLaz*, belong to the lipocalin family (35) and are predicted to carry small hydrophobic molecules such as steroids, bilins, retinoids and lipids (36). Both are expressed in glial cells in the developing and adult nervous system (37–39). Recent studies in plants, flies and mice indicate an important function for ApoD proteins as antioxidant defenses. Loss of function mutants showed increased sensitivity to oxidative stress (39–41). In contrast, overexpression enhanced resistance to oxidative stress and increased lifespan under normal conditions (41–43).

In this study, we address whether frataxin depletion in *Drosophila* results in an abnormal lipid metabolism and whether such metabolic dysfunction is an important element for the development of the disease. In addition, we hypothesized that *GLaz* might be an important candidate modulating the frataxin-deficient phenotype. We report that reduction of *Drosophila* frataxin (*fh*) expression either ubiquitously or in a pan-glial pattern produced a massive accumulation of FAs. In addition, loss of glial frataxin resulted in shortened lifespan, increased sensitivity to oxidative stress, impaired locomotor activity and progressive vacuolization of glial enriched regions. Interestingly, *GLaz* was able to significantly counteract some phenotypes detected in frataxin-deficient flies such as shortened lifespan, compromised locomotor performance and reduced aconitase activity. More importantly, this work provides *in vivo* evidence supporting a central role of lipid peroxides in the pathophysiology of FRDA.

RESULTS

Lack of frataxin in glial cells induces accumulation of lipid-like droplets

Although demyelination has been reported in post-mortem analysis of FRDA cases (44) and in two different FRDA mouse models (45,46), little is known about a possible glial dysfunction in FRDA neuropathology. Invertebrate glia do not generate myelin sheaths; however, they form multi-layered membrane protections around the neurons. To investigate these aspects in a *Drosophila* model system, expression of *fh* was downregulated in glial cells by means of the UAS-GAL4 system and the use of the pan-glial *Repo*-GAL4 driver. This driver was described by Sepp *et al.* (47) as a glial-specific GAL4 line expressed in all ectodermally derived glia within the PNS and CNS, reproducing the endogenous *Repo* expression (48).

Selective interference of frataxin in glial cells at 25°C using *fhRNAi-1* produced viable offspring as well as some pre-adult lethality. The brains of these flies were examined in semi-thin plastic sections stained with toluidine blue by light microscopy. We found that when *fh* was specifically knocked-down in glial cells, frataxin-deficient flies showed abundant droplet-like structures throughout the entire brain cortex (Fig. 1B and C). The locations of these droplets correlate well with the staining pattern for anti-Repo observed in a wild-type brain (data not shown).

In order to analyze the structure of these droplets, the brains of *fhRNAi-1* flies were further studied by electron microscopy. The ultrastructural examination revealed that the cell bodies of glial cells were densely packed with lipid-like vesicles (Fig. 1E) where the number and size progressively increased during aging (data not shown). To determine the lipidic composition of the droplets found in glia cells, we analyzed the FA content of *Drosophila* brains from 35-day-old flies by means of the gas chromatography-mass spectrometry (GC/MS) technique. We found that loss of frataxin in glial cells resulted in a robust accumulation of some FAs. Specifically, myristic (C14:0) and palmitoleic (C16:1) acids were highly increased, whereas oleic acid (C18:1) only showed a moderate increment (Fig. 1G).

These results suggest a disruption of lipid metabolism in glial cells triggered by loss of frataxin and the concomitant formation of multiple lipid droplets.

Frataxin knock-down increases free FA levels

Next, we wanted to characterize the lipid accumulation in a scenario that better resembles patient conditions (systemic frataxin deficiency). In order to elucidate this question, frataxin was ubiquitously reduced with daughterless-GAL4 (*da*-GAL4), and subsequently these flies were then subjected to a semi-quantitative analysis of lipid content using thin-layer chromatography.

In our hands, a strong systemic reduction of frataxin using *fhRNAi-1* produced mainly long-lived L3 larvae, as described by Anderson *et al.* (49), and just a few of them were able to pupate. This second group of larvae was used to carry out the following experiments.

As shown in Figure 2, a 65% increase in the free FA content could be detected compared with control larvae (*da*-GAL4/+), whereas the TAG only showed a small 10% increase
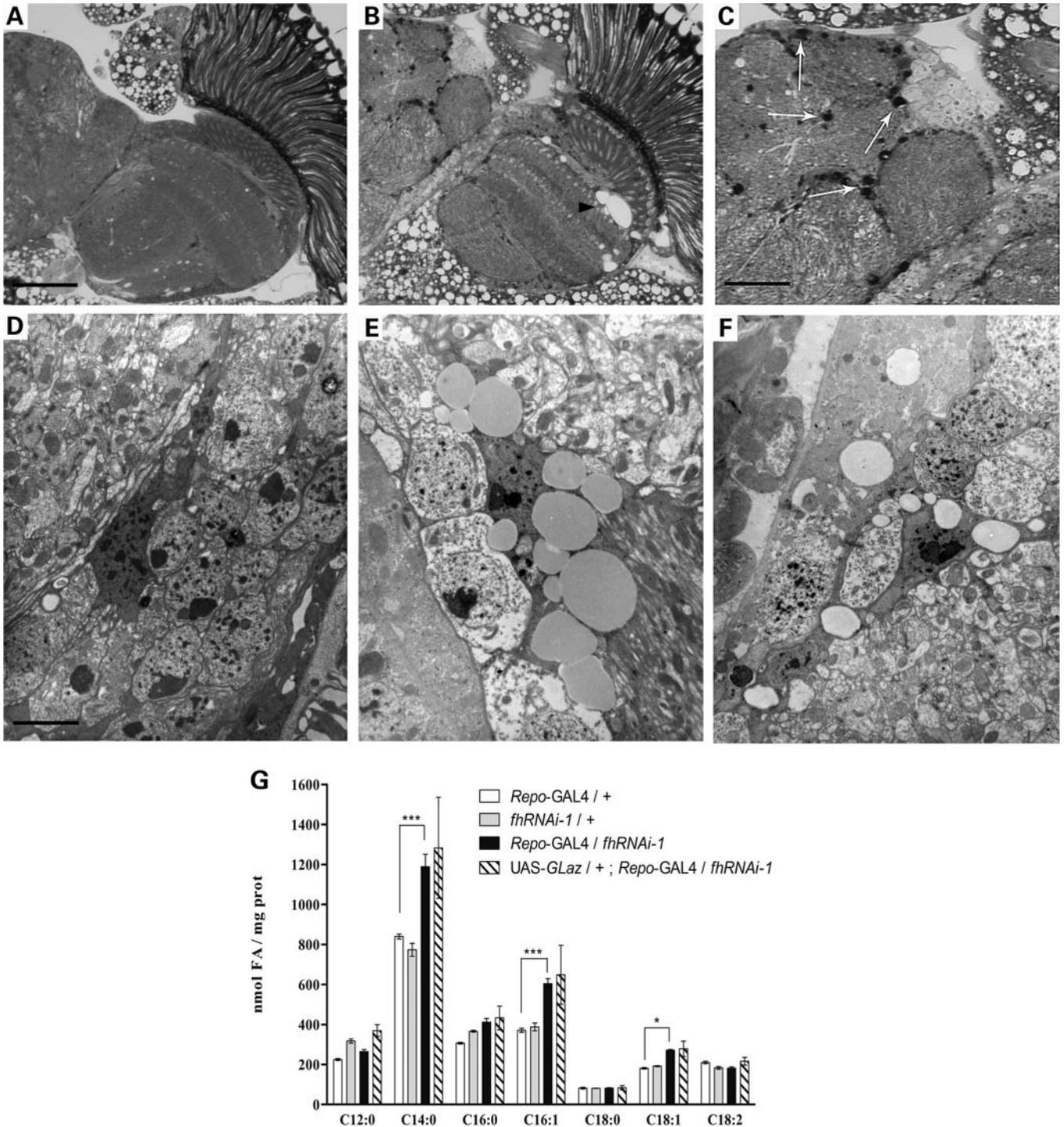
Figure 1. Lipid droplet accumulation in frataxin-deficient glial cells. (**A** and **D**) 35-day-old *Repo*-GAL4/+ controls. (**B**, **C** and **E**) 35-day-old *Repo*-GAL4/ *fhRNAi-1*. (**F**) 35-day-old UAS-*GLaz*/+;*Repo*-GAL4/*fhRNAi-1*. (B) Frataxin-deficient brains displayed degenerative defects in the form of extensive outer chiasm vacuolization (arrowhead). (C) The appearance of lipid-like droplets is denoted with white arrows. (D and E) Ultrastructural analysis revealed accumulation of lipid bodies in glial cells of frataxin-deficient brains. (F) Altered properties of glial-lipid deposits after co-expression of *GLaz*. (**G**) GC/MS analysis of FAs from *Drosophila* heads. Frataxin deficiency results in a remarkable increase of C14:0, C16:1 and C18:1. *GLaz* is unsuccessful in restoring the amount of these three FAs to control levels. Significance in (G) was determined by one-way ANOVA with *post hoc* Newman–Keuls (***$P < 0.001$ and *$P < 0.05$). Error bar represents standard error. FA, fatty acid. The scale bar represents 50 μm (A and B); 25 μm (C) and 2.5 μm (D–F).

(Fig. 2A). The level of different phospholipids [phosphatidylcholine (PC), phosphatidylglycerin (PG) and phosphatidylinositol (PI)] did not show any relevant increase (Fig. 2B).

In order to determine whether such an accumulation was due to a general increase of FAs or only to a contribution of some specific species, we carried out a GC/MS analysis of FAs.
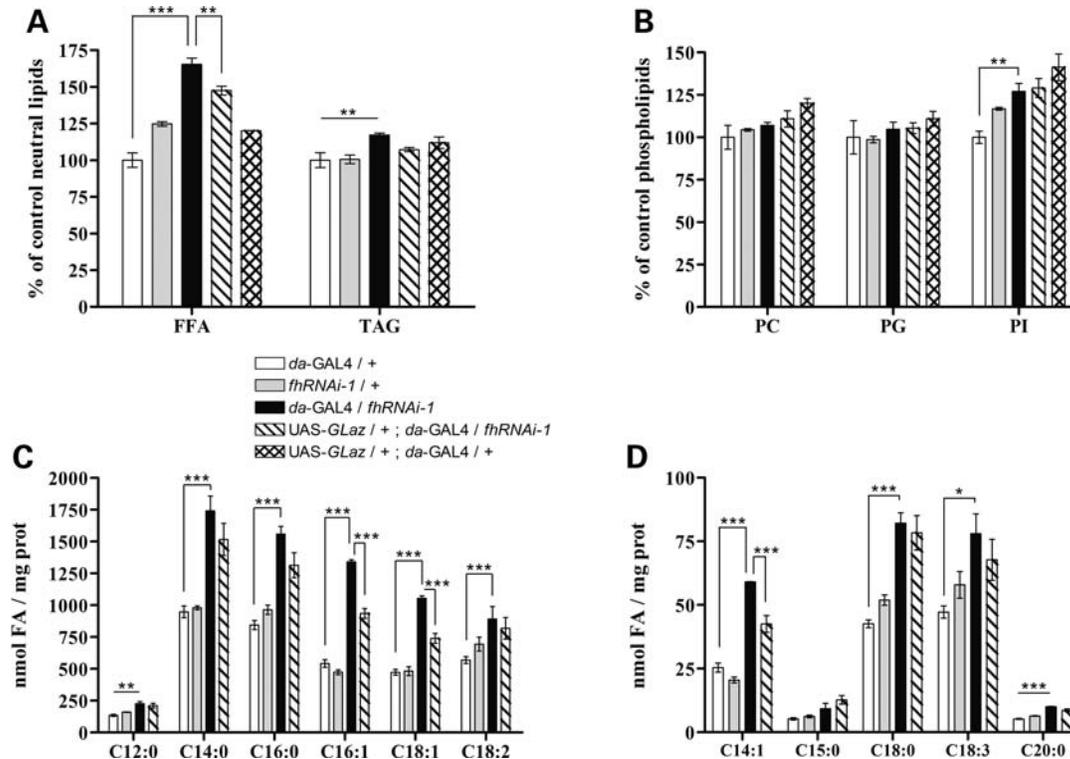
Figure 2. Lipid analysis of frataxin interference lines detects a drastic increase in free FAs partially rescued by *Drosophila GLaz*. (**A** and **B**) Analysis of neutral and phospholipid levels by thin-layer chromatography in late L3 larvae. The values are expressed as a percentage of control (*da*-GAL4/+). Frataxin knock-down (*da*-GAL4/*fhRNAi-1*) mainly triggers accumulation of free FAs when compared with controls. These accumulations are partially rescued by *GLaz* co-expression (UAS-*GLaz*/+;*da*-GAL4/*fhRNAi-1*). (**C** and **D**) GC/MS analysis of FA content. (C) Comparison of the most abundant FA in our samples. (D) Comparison of low-level FA (<10%). Significance was determined by one-way ANOVA with *post hoc* Newman–Keuls (***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$). Error bar represents standard error. FFA, free fatty acid; TAG, triacylglycerides; PC, phosphatidylcholine; PG, phosphatidylglycerin; PI, phosphatidylinositol; FA, fatty acid.

In the L3 samples, we were able to detect a profile consisting of 19 different FAs. Eight of them were extremely low represented (<6‰) and were not considered in our study. Myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1) and linoleic acid (C18:2) were the most abundant acids (each of them representing >10% of total FA composition). In addition, FAs with chains containing more than 20 carbons were not detected. Figure 2C and D shows that frataxin deficiency induced a general accumulation of FAs independently of their relative abundance compared with control flies. In agreement with the glial results (Fig. 1G), the increase was higher in the saturated and monounsaturated FAs (from 65 up to 160%) in contrast to only slight increases detected in the polyunsaturated FAs (25%).

Taken together, these results suggest that loss of frataxin triggers a general accumulation of FAs either by an increased synthesis or by a defective catabolism. This finding is in agreement with our observation of accumulation of lipid-like droplets in glial cells of frataxin-deficient flies.

Overexpression of *GLaz* counteracts the accumulation of FAs in frataxin knock-down flies

GLaz is an important protein involved in antioxidant defense and lipid metabolism in glial cells and therefore a good candidate for attenuating the frataxin-dependent glial phenotype. A first hint for such an interaction came from the ultrastructural

analysis, which shows a modification of the quality of lipid droplets in the glial cells of frataxin knock-down flies that co-express *GLaz* (compare Fig. 1E with F). In agreement with this observation, lipid analysis of L3 larvae that ubiquitously co-expressed *GLaz* showed a significant reduction of free FA content but no changes in TAG or phospholipids (Fig. 2A and B). To study any specific effects of *GLaz* on FA content, a GC/MS approach was performed identifying a biased action of *GLaz* with an exclusive and partial recovery of monounsaturated FAs, but not saturated or polyunsaturated FAs (Fig. 2C and D).

To exclude that the partial rescue of *GLaz* is due to a GAL4 dilution effect using multiple UAS constructs in one genotype, expression levels were compared by semi-quantitative real-time PCR. Both genotypes, single frataxin interference (*da*-GAL4/*fhRNAi-1*) and co-expression of *GLaz* (UAS-*GLaz*/+;*da*-GAL4/*fhRNAi-1*) showed a similar frataxin level of ∼10% relative to control values (data not shown).

Our results clearly indicate that *GLaz* is capable of partially rescuing the frataxin lipid phenotype by selectively reducing the increased amount of monounsaturated FAs.

Glial function of frataxin in lifespan and oxidative stress

Next, we investigated the impact of the glial function of frataxin on fly survival. Enhanced sensitivity to oxidative insult is a hallmark in FRDA models, hence we examined whether
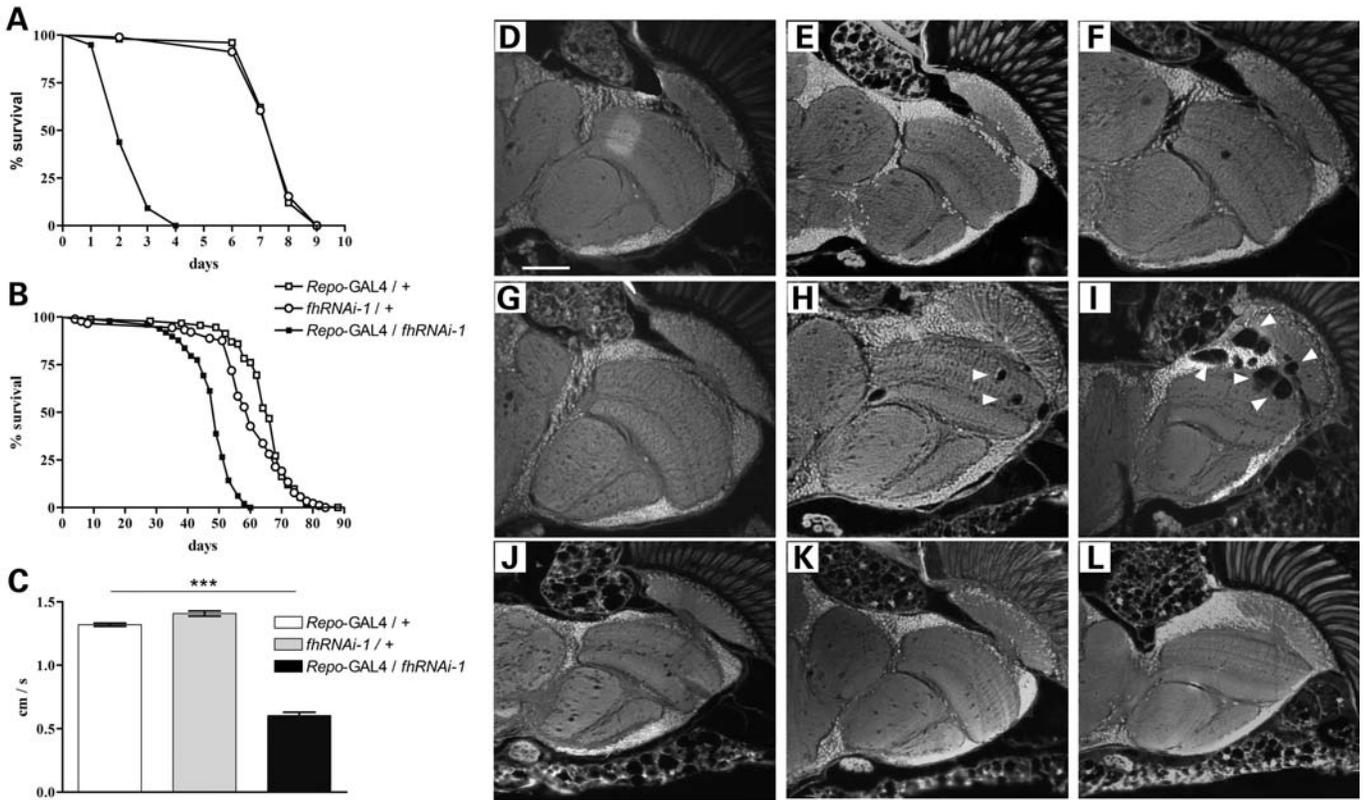
Figure 3. Physiological and behavioral effects of glial frataxin depletion. (**A**) Lifespan under hyperoxia (99.5% O_2). Downregulation of frataxin in glia strongly enhances susceptibility to oxidative stress. (**B**) Lifespan under normoxia. Pan-glial reduction of *fh* expression shortens the mean and maximum lifespan compared with control flies. (**C**) Negative geotaxis experiment with 10-day-old individuals. Loss of frataxin in glia strongly reduced walking ability. (**D–L**) Autofluorescent paraffin brain sections. (D–F, *Repo*-GAL4/+controls); (G–I, *Repo*-GAL4/*fhRNAi-1*) and (J–L, *fhRNAi-1*/+controls). (D, G and J, 5-day-old flies); (E, H and K, 20-day-old flies) and (F, I and L, 35-day-old flies). Frataxin downregulation in glial cells induces age-dependent brain vacuolization focused on the outer chiasm, lamina and medulla (arrowheads). Statistical differences between survival curves in (A) and (B) were analyzed using the Kaplan–Meier test, and exclusively *Repo*-GAL4/*fhRNAi-1* showed a statistical significant behavior ($P < 0.001$). Significance in (C) was determined by one-way ANOVA with *post hoc* Newman–Keuls (***$P < 0.001$). Error bar represents standard error. The scale bar represents 50 μm.

reducing frataxin expression in glial cells was sufficient to exacerbate the sensitivity to oxidative stress. Hyperoxia has been proven to be a relevant system to re-create an unbalanced oxidative status (50). As can be seen in Fig. 3A, frataxin interference (*Repo*-GAL4/*fhRNAi-1*) under oxygen-induced stress resulted in a drastic reduction of mean (73%) and maximum lifespan (55%) when compared with controls. Shortening of mean (25%) and maximum (32%) lifespan was also revealed by survival analysis under normal conditions (Fig. 3B).

These results indicate that frataxin is essential for a correct function of glial cells. In particular, absence of glial frataxin increased sensitivity of this cell type towards reactive oxygen species (ROS), leading to premature death of the flies.

Depletion of frataxin expression in glial cells affects nervous system integrity

In order to study the effect of frataxin downregulation on the integrity and function of the *Drosophila* nervous system, locomotor activity and brain degeneration of *Repo*-GAL4/*fhRNAi-1* flies were analyzed.

Loss of frataxin provoked a 55% reduction in the climbing ability of 10-day-old flies (Fig. 3C). Moreover, brain analysis

showed an age-dependent degeneration that was first detected at day 20 and further increased at day 30 (Fig. 3G–I). The vacuolization is mainly focused on medulla, lamina and outer chiasm (Fig. 3I, arrowheads), which is consistent with a location of tissue enriched in glial cells. In addition, an ultra-structural analysis showed that the lipid droplets described in Figure 1 can be detected before the presence of vacuolization (data not shown).

Our results suggest that lack of frataxin initiates a glial dysfunction that correlates with the decrease in negative geotaxis performance concomitant with a lipid accumulation finally culminating in cell death.

GLaz overexpression partially rescues loss of frataxin phenotypes in glial cells

Next, we examined the rescue efficiency of *GLaz* in glia. As shown in Figure 4A and B, *GLaz* was able to clearly extend the lifespan of frataxin-deficient flies under oxidative stress as well as under normal conditions (50 and 25%, respectively). Furthermore, *GLaz* could significantly improve the climbing performance of the affected individuals at 5 and 10 days (Fig. 4C). The same behavioral experiments were conducted
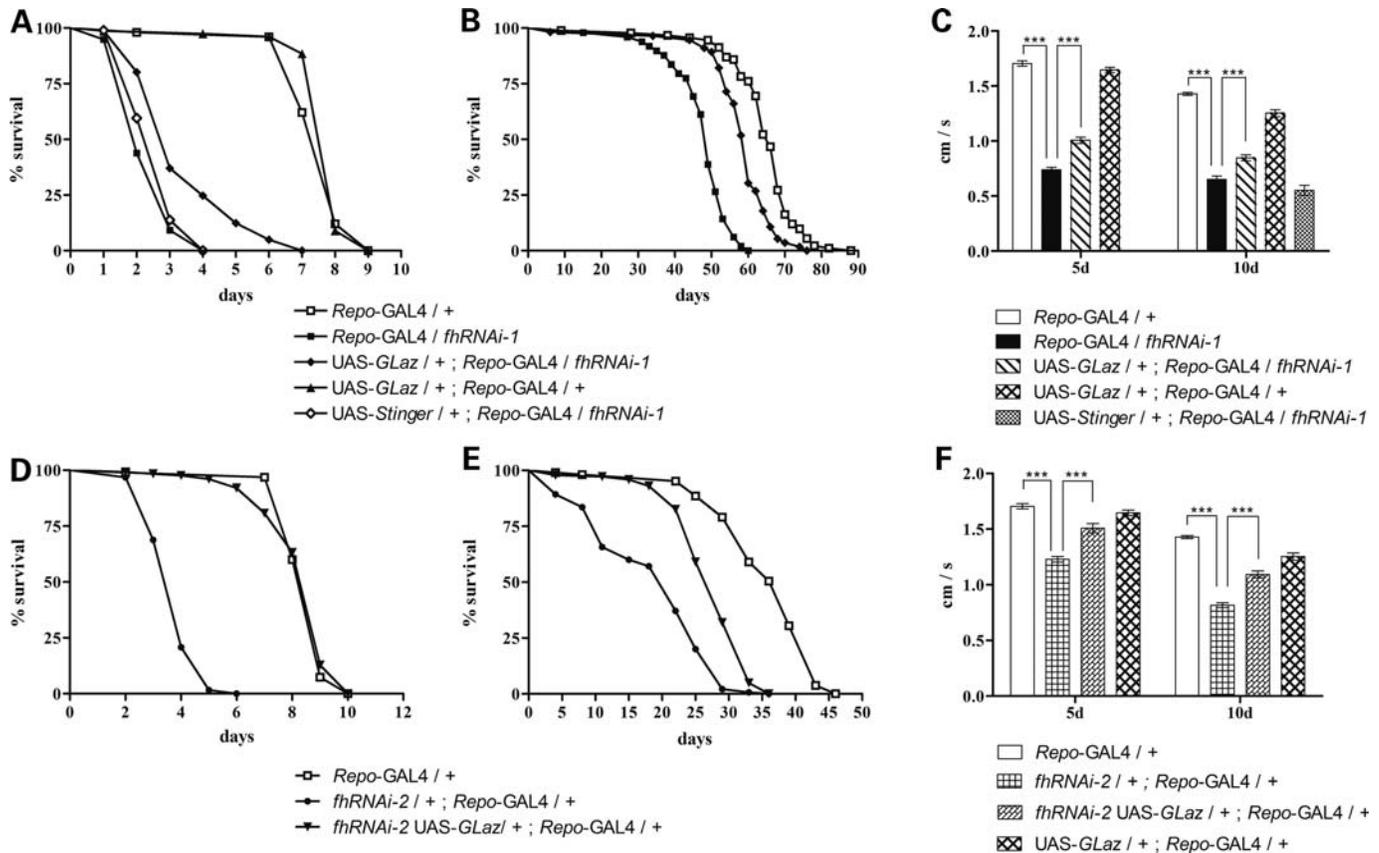
Figure 4. Lifespan and behavioral rescue of *fh*-deficient *Drosophila* by *GLaz* overexpression. (**A** and **D**) Lifespan experiments under hyperoxia conditions (99.5% O_2) using either *fhRNAi-1* (A) or *fhRNAi-2* (D) flies. *GLaz* is able to partially protect frataxin-deficient *Drosophila* against oxidative insult although no benefit is detected in control flies. (**B** and **E**) Lifespan under standard conditions using *fhRNAi-1* (B) or *fhRNAi-2* (E) flies. Co-expression of *GLaz* shows an extension of mean and maximum lifespan of flies with reduced frataxin. (**C** and **F**) Negative geotaxis experiments of 5- and 10-day-old flies using *fhRNAi-1* (C) or *fhRNAi-2* (F) flies. *GLaz* alleviates the locomotor deficits in frataxin knock-down individuals. Survival curves were analyzed using the Kaplan–Meier test. Significance in (C) and (F) was determined by one-way ANOVA with *post hoc* Newman–Keuls (***P < 0.001). Error bar represents standard error.

in *fhRNAi-2* flies, which showed similar results, although with a stronger *GLaz* rescue effect (Fig. 4D–F).

In order to exclude possible GAL4 dilution artifacts due to the presence of a second UAS line, negative geotaxis experiments and survival under hyperoxia were also carried out with co-expression of a nuclear GFP construct (UAS-*Stinger*). No significant differences were observed in both experiments compared with frataxin interference (Fig. 4A and C).

Next, we asked whether *GLaz*, when overexpressed in glial cells, is also able to partially rescue the FA phenotype induced by a reduction of frataxin levels. By means of the GC/MS technique, we found that *GLaz* failed to reduce the amount of the monounsaturated FAs C16:1 and C18:1 in the head samples (Fig. 1G). As can be seen in Figure 1F, the ultrastructural analysis by electron microscopy of UAS-*GLaz*/+;*Repo*-GAL4/*fhRNAi-1* brains also displayed the vacuolization and the lipid vesicles observed in *Repo*-GAL4/*fhRNAi-1* individuals (Fig. 1E), with the peculiarity that the vesicles seemed empty after preparation. De Martino *et al*. (51) carefully studied the influence of distinct plastic embedding media and staining procedures on the lipid morphology in light and electron microscopy and they discovered that the binding affinity of osmium tetroxide varies proportionally with the oxidation status of the FAs.

This would suggest that *GLaz* function has an influence on the nature of the lipids stored in the vesicles and that the oxidation state of these lipids might be altered by the co-expression of *GLaz* in glial cells.

These results show that *GLaz* is able to increase the lifespan and the locomotor ability of frataxin-deficient flies without directly reducing the excess of FAs in the glial cells and therefore indicating that there is not necessarily a direct link between lipid accumulation and behavioral phenotypes.

Reduction of frataxin expression promotes lipid peroxidation

Human ApoD and *Drosophila GLaz* have been reported to repress lipid peroxides (39,41,43). Moreover, lipid peroxidation products such as MDA have been regularly found in FRDA patients' samples (18). Therefore, we asked whether frataxin interference also increased lipid peroxidation levels in *Drosophila*. In order to elucidate the mechanism by which *GLaz* improved frataxin-deficient conditions, we studied the effect of systemic frataxin reduction on lipid peroxidation. As reported in Llorens *et al*. (15), a moderate ubiquitous reduction of frataxin (*fhRNAi-2*) was enough to bypass any pre-adult lethality and shows some typical features of FRDA, such as
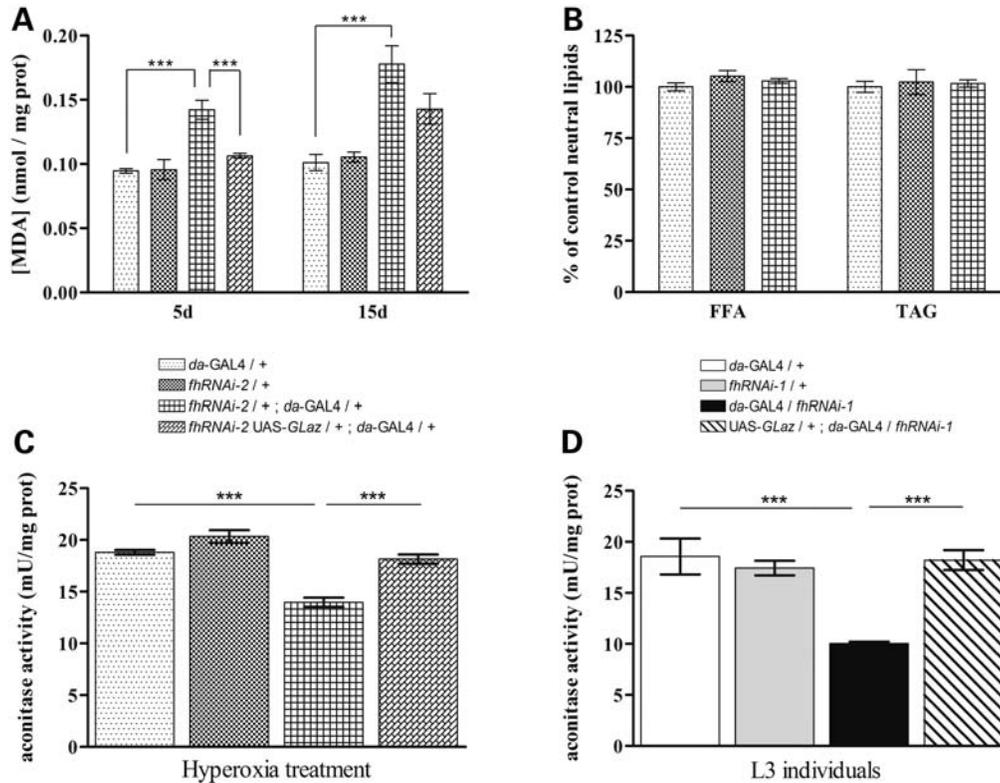
Figure 5. *GLaz* decreases lipid peroxidation levels and restores aconitase activity in frataxin-deficient flies. (**A**) Frataxin-deficient adults (*fhRNAi-2/+*; *da-GAL4/+*) display an increase in lipid peroxides that can be restored to control levels by *GLaz* in 5-day-old flies (5d) but not in 15-day-old flies (15d). (**B**) *fhRNAi-2/+*;*da-GAL4/+* do not induce any significant changes in TAG or FFA content. (**C** and **D**) *GLaz* significantly protects aconitase from ROS-mediated inactivation in frataxin-deficient L3 (caused by *fhRNAi-1* transgene) and adult flies (caused by *fhRNAi-2* transgene). Significance was determined by one-way ANOVA with *post hoc* Newman–Keuls (***$P < 0.001$). Error bar represents standard error. FFA, free fatty acid; TAG, triacylglycerides.

enhanced sensitivity to oxidative stress, together with reduced aconitase activity and shortened lifespan.

As shown in Figure 5A, the total amount of the MDA product was notably increased in 5- and 15-day-old frataxin-deficient flies. Nevertheless, neither young (7-day-old, Fig. 5B) nor older (30-day-old, data not shown) frataxin-deficient flies present significant alterations in the TAG or free FA content. Interestingly, co-expression of *GLaz* showed an almost complete rescue of this phenotype, with a strong reduction (30%) of lipid peroxide accumulation in young flies (5-day-old). A mild decrease (9%) was observed in older individuals (15-day-old).

These results suggest that lack of frataxin besides inducing hypersensitivity to oxidative insult also increases the production of oxidative radicals by itself. Our data also indicate that the abnormal peroxidation of lipids in frataxin knockdown flies is independent of lipid accumulation. In addition, *GLaz* is able to reduce the increased lipid peroxide generation, although in a time-restricted manner. Therefore, the increase in lipid peroxidation correlates well with the phenotypes described in glia, and *GLaz* was able to rescue the effects driven by the loss of frataxin.

GLaz restores aconitase activity in frataxin-deficient larvae and adult flies

One of the most characteristic biochemical defects associated with a loss of frataxin is the reduction of aconitase activity

(9,15,33). Therefore, we asked whether co-expression of *GLaz* was able to restore the enzymatic activity in a frataxin-deficient environment. This hypothesis was tested in two different experimental scenarios: on the one hand, in L3 larvae with a strong reduction of frataxin produced by the *fhRNAi-1* transgene (17); on the other hand, in young adult flies with a milder reduction of frataxin caused by the *fhRNAi-2* transgene combined with oxidative stress (15). We used the *da*-GAL4 driver because it provides a more widespread expression facilitating the detection of changes in aconitase activity. Loss of frataxin provoked a 28% decrease in aconitase activity in hyperoxia-treated adult flies (Fig. 5C) and 46% in larvae (Fig. 5D). *GLaz* was able to restore aconitase activity effectively, up to levels comparable with control flies (Fig. 5C and D).

Taken together, these data indicate that preserving the redox balance is a major mechanism by which *GLaz* co-expression is able to counteract some of the effects caused by frataxin deficiency.

DISCUSSION

FRDA, the most frequent form of hereditary ataxia in the Caucasian population, is produced by a deficit in the mitochondrial protein frataxin. Although big efforts are being made using animal, cell culture and yeast models, the function of frataxin still remains elusive and controversial. Nevertheless, using

microarray and proteomic approaches, new and interesting downstream consequences derived from frataxin depletion are currently being described. These pathways are suggesting novel therapeutic targets for future treatments designed to mitigate lack-of-frataxin phenotypes (52).

In this work, we could show that a reduction of frataxin expression compromised the lipid homeostasis at two different levels: increasing the total amount of lipids and enhancing the generation of peroxyl radicals. Our experiments revealed that strong ubiquitous *fh* interference with *fhRNAi-1* triggered an accumulation of FAs in larvae. We also showed that a general reduction of frataxin to levels compatible with a normal development provoked an early overload of lipid peroxides without a detectable rise in the level of FAs. Thus, the increase in lipid peroxides is probably due to a higher ROS production rather than to an FA accumulation. However, we can speculate that the increase of FA mediated by a strong reduction of frataxin expression could exponentially increase the oxidation rate of FAs.

The FA accumulation observed in the *Drosophila* FRDA model is in good agreement with the lipid inclusions found previously in the cardiac muscle of FRDA conditional mouse models (33) and with the increased intracellular lipid content present in the striated muscle fibers and Schwann cells of inherited α-tocopherol deficiency patients (21), a disease that appears to be clinically indistinguishable from FRDA. Moreover, up to 40% of FRDA patients manifest different glucose metabolism problems (53) such as diabetes or insulin resistance, and remarkably type 2 diabetes patients also present an altered lipid status attributable to a mitochondrial dysfunction (54,55). However, the link between increased lipid amounts and FRDA is not completely clear. On the one hand, depletion of frataxin generates different scenarios that can lead to the inhibition of mitochondrial β-oxidation. For example, mitochondrial respiration defects induce a reduction of $NAD^+/NADH$ ratios compromising the β-oxidation pathway (22,56). Alternatively, impairment of aconitase activity increases citrate levels (57), and citrate is an allosteric activator of malonyl-CoA production, which in turn is a potent inhibitor of the mitochondrial β-oxidation (reviewed in 58). Furthermore, downregulation of peroxisome proliferator-activated receptor gamma (PPARγ) pathway has been observed in FRDA mice models, suggesting a reduction of lipid catabolism (59). Moreover, on the other hand, frataxin deficiency has been suggested to increase lipogenesis via hyperactivation of mitochondrial acyl-CoA thioesterase (60) or upregulating the expression of the sterol-responsive element-binding protein 1 (Srebp1) (59). Thus, derangement of lipid homeostasis in FRDA could be produced by either blocking the main degradation pathway of FAs or increasing their synthesis. In addition, lipid imbalance could be a critical event in FRDA progression since lipid metabolism is the main fuel source of the cell.

FAs are extremely sensitive to oxidative modification, resulting in the formation of lipid peroxides, which are highly cytotoxic and promote damage to other lipids, proteins and DNA (reviewed in 61). The link between oxidative stress and frataxin has been extensively analyzed. Indeed, loss of frataxin not only promotes hypersensitivity to oxidative insult in various experimental models (12–17), it also induces accumulation of oxidative stress markers such as anion superoxide (62–64), carbonylated proteins (16,65) and MDA or other lipoperoxidation products (46,66). Moreover, frataxin overexpression reduces endogenous levels of ROS in cell culture (67) and increases antioxidant activity in *Drosophila* (68). The age-dependent accumulation of lipid peroxides found in our *Drosophila* model corroborates the endogenous increase in ROS production led by loss of frataxin. Castilho *et al.* (69,70) reported that lipid peroxides trigger a cascade of events that includes a fall in mitochondrial membrane potential, mitochondrial swelling and loss of mitochondrial matrix components. More interestingly, lipid peroxidation is strongly catalyzed through the Fenton reaction by iron and iron complexes (71,72), being the iron–citrate complex one of the most toxic forms. Remarkably, iron is the transition metal that accumulates in frataxin-deficient yeast and in the mitochondria of different samples from FRDA patients (6,11,73). Thus, iron-mediated oxidative insult seems to develop a crucial role in the function of frataxin and in the progression of the disease. Since lipid peroxides are the most harmful oxidative stress mediators, strategies focused on quenching, recycling or protecting such products could be highly beneficial for the treatment of the disease.

The lipid metabolism failure is not restricted to a systemic reduction of frataxin. When *fh* expression is suppressed in glial cells, an accumulation of FAs was first detected with electron microscopy and confirmed by gas chromatography. Since neuron–glia interactions are crucial to ensure optimal brain function and glia are the principal neuron energy suppliers, our results may indicate a pivotal function of glial cells in the FRDA neuropathology. Indeed, posterior nerve roots and posterior sensory nerves in FRDA patients are characterized by an axonal atrophy as well as by a demyelination process, whereas the fine unmyelinated fibers are well preserved (44,74). Interestingly, Lu *et al.* (75) have recently reported that frataxin knock-down produced severe effects on Schwann cell cultures compared with DRG and with other neuronal cell lines. However, despite this pathological condition, the role of glial cells in the FRDA etiology has not yet been analyzed in detail. In this work, we report that the reduction of frataxin expression in *Drosophila* glial cells induces adverse effects such as a hypersensitive response against oxidative insult, a decrease in locomotor performance and shortened lifespan. Moreover, we present the first evidence of nervous system degeneration in an invertebrate model of FRDA. Interestingly, some phenotypes were detected prior to a clear lipid accumulation. Therefore, together with the results from the ubiquitous frataxin interference, we suggest that frataxin depletion in glial cells leads to increased lipid peroxidation that induced mitochondrial injury and dysfunction preceding the lipid accumulation and cell degeneration. Although we have not studied this aspect, alterations in glial cells might also occur in the PNS, contributing to the glial phenotypes described in this work.

If lipid dysregulation is a significant factor for FRDA pathology in *Drosophila* and lipid peroxides are a primary cause of the symptoms, we might be able to mitigate loss-of-frataxin effects by modulating the lipid content and/or the amount of lipoperoxides. *GLaz*, one of the *Drosophila* homologs of human ApoD, is expected to counteract both conditions (39,42,76). In frataxin-deficient flies, *GLaz*, indeed, exhibited

cell-specific effects. First, general co-expression of *GLaz* specifically reduced the amount of monounsaturated FAs. In agreement with these results, ApoD and its homolog proteins have been shown to regulate systemic lipid homeostasis in mice (77) and *Drosophila* (78), as well as to control the brain lipid composition in mice (79). Our results would also suggest that oleic acid (C18:1), palmitoleic acid (C16:1) and myristoleic acid (C14:1) are reliable biological ligands of *GLaz*. These data correlate with the *in vitro* binding properties described for grasshopper Lazarillo (76). Second, ubiquitous co-expression of *GLaz* also decreased the level of lipid per-oxides in frataxin knocked-down flies. Although levels of lipid peroxidation markers are highly increased in *GLaz* null mutants (39), it is not yet clear whether *GLaz* is able to bind lipid peroxidation products or, alternatively, is able to prevent lipid oxidation from occurring. However, all lipocalin family members are thought to bind small hydrophobic mol-ecules due to their common tertiary structure (35) and some lipocalins such as human lcn-1 act directly on peroxidated lipids (80), pointing to a lipid-scavenging mechanism for *GLaz*. In addition, the plant ApoD ortholog knock-out also showed accumulation of other ROS (40). Third, restoration of lipid peroxides and other free radicals to normal levels by *Glaz* overexpression in frataxin-deficient flies appeared to be sufficient to prevent mitochondrial aconitase inactivation. Similarly, ectopic expression of hydrogen peroxide scavengers such as mitochondrial catalase or mitochondrial peroxiredoxin was able to extend lifespan of flies with reduced frataxin in the PNS. In addition, aconitase activity was restored using mito-chondrial catalase in this *Drosophila* model of FRDA (17). Aconitase activity has also been rescued using the antioxidant compound idebenone in heart homogenates from FRDA patients (66) and by means of iron quelator molecules such as deferiprone (81) and mitochondrial ferritin (82) in frataxin-silenced mammalian cell cultures. These results would indicate that loss of aconitase activity in frataxin-deficient conditions is basically due to an imbalanced redox status in the cell and would suggest a role for frataxin in oxidative stress control. Some authors have proposed that frataxin carries out such tasks through a ferroxidase activity that detoxifies mitochondrial iron and prevents oxidative damage (83,84) or by acting as an iron sensor to negatively regulate the synthesis of Fe−S clusters (85), preventing the generation of oxidative damage from iron deposits and/or misfolded Fe−S clusters. Whereas, others have linked frataxin deficiency with impaired recruitment of antioxidant defenses (86−88).

Finally, co-expression of GLaz in the glia of frataxin-deficient flies is sufficient to produce at least a partial recovery of lifespan in both normoxic and hyperoxic conditions, as well as to cause significant improvements in locomotor activity, indicating that its modulation of lipid composition, lipid oxi-dation and aconitase activity do result in improvements in the physiological state of the whole organism. Human ApoD has been found to be highly upregulated in many neurological diseases or after nervous tissue damage (reviewed in 34,89); however, little is known about its abilities as a neuroprotective molecule. Beneficial effects of exogenous human ApoD have been reported after the induction of encephalitis in mice (90) and cytotoxic injury in hippocampal rat cultures (91). In both cases, the proposed mechanism implies the disposal of

lipid peroxides and regulation of inflammatory responses. Remarkably, frataxin deficiency in Schwann cell cultures also triggered the upregulation of inflammatory genes (75). Therefore, we propose that, in our FRDA model, *GLaz* mainly exerts its protective function by ROS scavenging via quenching lipid peroxides.

Our results may suggest that initial aconitase failure is mostly due to the exposure to increased oxidative stress rather than to the lack of Fe−S clusters. We suggest that lack of frataxin alters the efficient production of these clusters. This dysregulation triggers a cascade of oxidative stress via iron-toxic precipitates, lipid peroxidation and H_2O_2 overpro-duction, that quickly would start to inactivate mitochondrial aconitase. This hypothesis would explain why antioxidant pro-teins are able to restore aconitase activity. However, reactiva-tion of aconitase activity via *mitochondrial catalase* (17) or *GLaz* (this work) was not sufficient to bypass pre-adult lethal-ity. This clearly indicates that other elements (for example, respiratory chain complexes) and/or other processes (namely, Fe−S production) are also affected and could not be rescued by these antioxidant mechanisms.

In the present study, we demonstrate for the first time that fra-taxin expression in glial cells is essential for normal development and adulthood in an *in vivo* animal model. Moreover, glia emerges as a crucial cell type for FRDA pathology. We also show that the control of lipid metabolism could represent a new strategy to improve some pathological conditions in patients. In addition, our results strongly support *GLaz* as a therapeutic agent in FRDA and in any neurodegenerative disorder in which redox imbalance is a major contributor to the pathophysiology.

Furthermore, our data promote the *Drosophila* glia model of FRDA as an excellent tool to study the mechanisms under-lying nervous system degeneration in the disease as well as to test the beneficial effects of new drugs or genetic rescues for different aspects of the nervous system function.

MATERIALS AND METHODS

Drosophila stocks

The *yw* and *w1118* strains were used as control lines in our experiments. P{*da.G32*-GAL4}, P{*Repo*-GAL4} and P{*UAS-Stinger*} were obtained from the *Drosophila* Bloo-mington Stock Center, and the line *UDIR1* was kindly pro-vided by John P. Phillips (University of Guelph). The stocks were routinely maintained on cornmeal agar medium at 25°C. The crosses of the GAL4 driver with the UAS responder lines were carried out at 25°C. UAS constructs were used in heterozygous configuration for all experiments. The lines *UDIR1* (49) and *UAS-fhIR* (15) have been renamed in the text and figures as *fhRNAi-1* and *fhRNAi-2*, respectively. *fhRNAi-1* induced a strong interference with a 90% reduction of frataxin expression, whereas *fhRNAi-2* produced a moderate 70% reduction compatible with a normal development. Rescue experiments were carried out first generating the stocks *UAS-GLaz*/CyO;*fhRNAi-1*/TM3 and *fhRNAi-2* UAS-*GLaz*/ CyO (in both lines, the presence of each construct was con-firmed by PCR). *UAS-Stinger*/CyO;*fhRNAi-1*/TM3 was also created and used as control. A recent issue concerning the targeted gene silencing in *Drosophila* is the influence of

off-target genes on the described phenotypes (92). Regarding FRDA fly models, the similar defects induced by both RNAi lines (*fhRNAi-1* and *fhRNAi-2*) and the observed rescues with *fh* overexpression (data not shown) virtually excludes the possibility of a false-positive result.

Semi-quantitative real-time PCR

Total RNA was extracted from 75 L3 larvae or 7-day-old adult individuals, using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. Using QuantiTect Rev. Transcription Kit (Qiagen GmbH, Hilden, Germany), 1 μg RNA was converted into cDNA and then used for real-time PCR reactions with QuantiTect SYBR Green RT–PCR Kit (Qiagen GmgH) on Roche's lightcycler system. *fh* expression was detected using FHRTFw: 5′AC ACCCTGGACGCACTGT3′ and FHRTRv: 5′CCAGGTTC ACGGTTAGCAC3′ primers and *GLaz* expression with *GLaz*Exon1FOR: 5′ATTTGCTGGGACAGATGCCTAC3′ and *GLaz*Exon2REV: 5′TGTTGTACGGCTCAAACTGAAA3′ primers. The Ribosomal protein 49 (*rp49*) was used as internal control and amplified using RP49RTFw: 5′CCAAGCACTT CATCCGCCACC3′ and RP49RTRv: 5′GCGGGTGCGCTT GTTCGATCC3′ primers. The results were analyzed with the lightcycler software (version 3.5) from Roche Molecular Bio-chemical, gene expression levels were referred to the internal control and the relative quantification was carried out by means of the ΔC_t method.

Lifespan and negative geotaxis assays

For lifespan assay, flies were collected within 24 h of eclosion and were raised at 25°C under a 12:12 h light/dark cycle and transferred to fresh food vials every 2–3 days. Hyperoxia treatment started 1 day post-eclosion and was performed by exposing flies in a glass container with a constant flux of 99.5% oxygen under a low positive pressure at 25°C. Flies were confined in groups of 25 and were transferred every day to new vials containing regular food. Climbing assays were performed as described in Botella *et al*. (93).

Brain histology

Adult flies were fixed with carnoy (ethanol:chloroform:acetic acid at a proportion of 6:3:1), dehydrated in ethanol and embedded in paraffin. Paraffin sections (7 μm) were analyzed under a fluorescence microscope.

For examination of adult fly brains with electron microscopy, ultrathin Epon plastic sections were post-stained with 2% uranyl acetate, followed by Reynolds' lead citrate and stabilized for transmission electron microscopy by carbon coating. Examination was done with a Zeiss EM10C/VR electron microscope at 80 kV. Glial cell material was identified by characteristically higher electron density.

Thin-layer and gas chromatography

Comparative measurement of neutral and phospholipid content was carried out using a thin-layer chromatography system from CAMAG Scientific, Inc.

Fifteen L3 male larvae or 80 male adult heads were placed in a Precellys cup containing 2.8 mm ceramic beads, frozen under liquid nitrogen and stored at −80°C until needed. Samples were then homogenized in 1 ml MQ water using a Precellys 24 homogenizer. Samples were cleaned from cellular debris by centrifugation at 2100*g* for 60 s. The protein content was determined from the cell homogenate by means of the Bradford assay. According to the procedure described by Bligh and Dyer (94), 500 μg of each sample were delipidated. Cellular lipids were separated on Silica Gel 60A Plates (Merck, Darmstadt, Germany) by high-performance thin-layer chromatography. Silica gel plates were run either with *n*-hexan:diethylether:acetic acid 80:20:1 (v/v) for neutral lipid quantification or with 2-propanol:methylacetat:-chloroform:methanol:0.25% KCl 25:25:25:10:9 (v/v) for phospholipid evaluation, stained with 10% cupric sulfate (in 8% phosphoric acid) and heated to 170°C for 20 min. Quantitation was achieved by densitometric scanning with a CAMAG TLC scanner II at 366 nm.

For gas chromatography coupled with mass spectrometry (GC/MS), 20 μg from each sample were subjected to FA methyl ester derivatization (J. Ecker unpublished data). Peaks were assigned using both the retention time and fragmentation pattern. Introduction of internal standards C13:0 (tridecanoic acid) and C21:0 *iso* permitted quantification.

For both experiments, all individuals were grown under controlled conditions. Parental crosses were allowed to lay eggs for 12 h in order to avoid deleterious effects of limited nutritional conditions.

Enzymatic assays

Total aconitase activity was determined from whole individuals, using Bioxytech Aconitase-340™ Spectrophotometric Assay Kit (OXIS International, Inc., Portland, OR, USA).

Twenty larvae or 25 adults were placed in a tube containing 500 μl of ice-cold homogenization buffer (0.2 mM sodium citrate and 50 mM Tris–HCl, pH 7.4) and gently pounded. The resulting mashes were centrifuged twice at 800*g* for 10 min at 4°C, discarding the pellet every time. Supernatants were disrupted by sonication for 30 s, with 1 s interval between each pulse. To measure the aconitase activity, 100 μl of each extraction was used. The aconitase activity of each genotype was measured from four independent samples and each extraction was measured twice. The aconitase activity was finally referred to protein amount using the Bradford assay (Bio-Rad Laboratories, Inc).

Lipid peroxidation assay

The concentration of MDA was determined using the LPO-586 assay (OXIS International, Inc., Portland, OR, USA) from 60 male flies. Triplicates of each genotype and age were measured and each replica was measured twice. The procedure was conducted as described in Sanchez *et al*. (39), with minor modifications.

Statistical analysis

Survival data were analyzed using the Kaplan–Meier (semi-parametric log-rank) test. Locomotor, chromatographic, lipid

peroxidation levels and aconitase activity data are presented as mean $\pm$ SEM of at least three replicates. Significance was determined by one-way ANOVA with *post hoc* Newman–Keuls multiple comparison test (***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$). All statistical analyses were carried out with the GraphPad Prism 4.0 software.

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