

Hydrogen peroxide scavenging rescues frataxin deficiency in a *Drosophila* model of Friedreich's ataxia

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Friedreich's ataxia (FRDA) is a neurodegenerative disorder arising from a deficit of the mitochondrial iron chaperone, frataxin. Evidence primarily from yeast and mammalian cells is consistent with the hypothesis that a toxic hydroxyl radical generated from hydrogen peroxide (H₂O₂) via iron-catalyzed Fenton chemistry at least partially underlies the pathology associated with this disease. However, no whole-organism studies have been presented that directly test this hypothesis. We recently developed a *Drosophila* model that recapitulates the principal hallmarks of FRDA [Anderson PR, Kirby K, Hilliker A, Phillips JP (2005) *Hum Mol Genet* 14:3397–3405]. Using the *Drosophila* FRDA model, we now report that ectopic expression of enzymes that scavenge H₂O₂ suppresses the deleterious phenotypes associated with frataxin deficiency. In contrast, genetic augmentation with enzymes that scavenge superoxide is without effect. Augmentation of endogenous catalase restores the activity of the reactive oxygen species (ROS)-sensitive mitochondrial enzyme, aconitase and enhances resistance to H₂O₂ exposure, both of which are diminished by frataxin deficiency. Collectively, these data argue that H₂O₂ is an important pathogenic substrate underlying the phenotypes arising from frataxin deficiency in *Drosophila* and that interventions that reduce this specific ROS can effectively ameliorate these phenotypes. The therapeutic implications of these findings are clear and we believe warrant immediate clinical investigation.

phenotypic rescue | catalase | Fenton chemistry | RNA interference

Friedreich's ataxia (FRDA) is a neurodegenerative disorder that arises from a genetic deficit of the mitochondrial iron chaperone protein, frataxin (1–3). The progressive loss of coordination of limb movements, dysarthria, nystagmus, scoliosis, and diabetes characteristic of FRDA usually manifest before adolescence. The peripheral nervous system (PNS) and the heart are among the most severely affected tissues, and more than half of those afflicted eventually succumb to cardiac-related complications (4–6).

Yeast, *Drosophila*, mouse, and cell culture models of frataxin deficiency have revealed important roles for frataxin and its homologs in mitochondrial iron storage (7–15), regulation of intracellular iron trafficking (7, 8, 10, 16, 17), iron-sulfur (Fe-S) cluster (16–21) and heme biogenesis (14, 22), and reactivation of the labile Fe-S cluster of mitochondrial aconitase (mACON) (22, 23). A role for frataxin in preventing formation of deleterious reactive oxygen species (ROS) has been well established (12, 15, 24), invoking a paradigm of FRDA pathology in which ROS toxicity leads to mitochondrial dysfunction with subsequent cell death (for review, see refs. 25 and 26).

The loss of intracellular iron chaperone capacity imposed by frataxin deficiency could elicit a Fenton chemistry-based mechanism of ROS toxicity. Elevated ROS production, including generation of the highly reactive hydroxyl radical, by iron-catalyzed Fenton chemistry with endogenous H₂O₂ as the substrate would be consistent with the susceptibility to H₂O₂ challenge noted in yeast and cell culture models of frataxin deficiency (3, 27–29). Yet no whole-organism animal studies

have been presented that investigate the role of H₂O₂ in mediating frataxin deficiency phenotypes.

We recently reported the development of a *Drosophila* model of FRDA (17) that takes advantage of Gal4/UAS transgene-based RNAi methodology to impose down-regulation of the *Drosophila* frataxin homolog (*Dfh*) (30). In this model, *Dfh* suppression recapitulates the principal biochemical hallmarks of FRDA, including diminished activity of Fe-S-containing enzymes, susceptibility to iron toxicity, loss of intracellular iron homeostasis, and early-onset adult mortality.

The hypothesis that H₂O₂ plays a critical role in FRDA pathogenesis predicts that interventions that diminish the availability of this potential reactant will reduce the severity of at least some aspects of the disease. We tested the validity of this hypothesis by using the *Drosophila* model to overexpress a set of H₂O₂-scavenging enzymes [peroxisomal and mitochondrial catalases (CATs) and a mitochondrial peroxiredoxin] in DFH-deficient flies. This report details the outcome of that investigation.

Results

The *Drosophila* FRDA model used in this work employs the *C96*-Gal4 driver to reduce endogenous DFH via RNAi and to coordinately augment native levels of antioxidant enzymes (17). As reported earlier, DFH deficiency imposed by expression of *UAS-DfhIR* transgenes controlled by the *C96*-Gal4 driver confers marked early-onset adult mortality that is reflected as a 40% reduction in media adult life span (17). The *C96*-Gal4 driver promotes robust expression in the PNS and oenocytes with low-level expression in many other tissues (A.J.H., unpublished data). Use of this driver favors, but is not exclusively restricted to, expression in one of the two principal focal tissues of FRDA. Unlike drivers that promote robust widespread expression, the more focused expression of *UAS-DfhIR* by *C96*-Gal4 permits vigorous development and viable but short-lived adults (17).

CAT Restores Life Span. To investigate the role of H₂O₂ in the pathophysiology of DFH depletion, we first asked whether augmentation of CAT would suppress the early-onset mortality that characterizes flies deficient in DFH. In *Drosophila*, CAT localizes to the cytoplasm/peroxisomal compartment, and transgenic augmentation of CAT is known to enhance resistance to oxidative stress (31). Using the Gal4/UAS transgene expression system, we augmented flies deficient in DFH with *Drosophila* CAT. It should be noted that because CAT augmentation is confined essentially to the peripheral nervous system, its activity is not detectable above endogenous CAT activity in whole-fly

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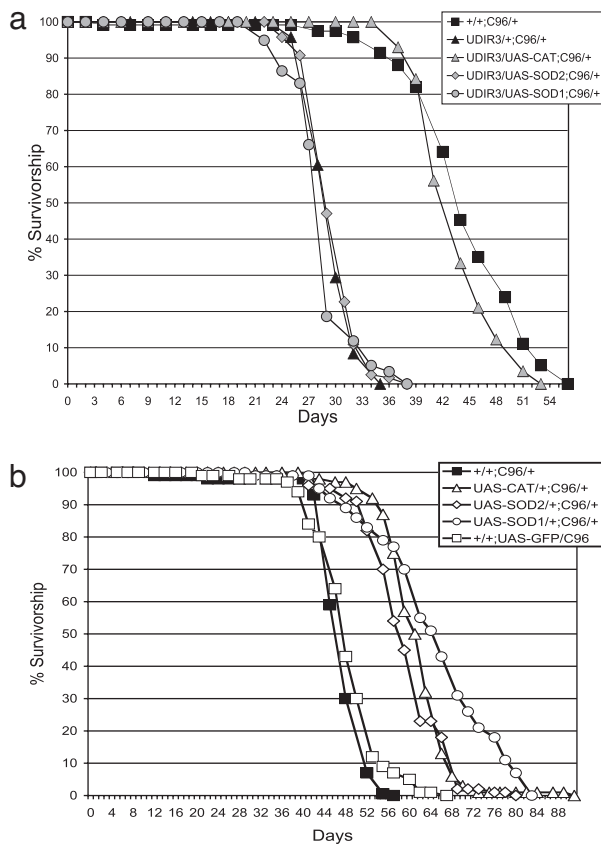


Fig. 1. CAT rescues life span in DFH-deficient *Drosophila*. (a) Augmentation of CAT, but not SOD1 or SOD2, restores life span in DFH-deficient *Drosophila*. (b) Expression of CAT, SOD1, and SOD2 in DFH-sufficient *Drosophila*. Survival of 125 males of each genotype on standard cornmeal food was followed at 25°C with enumeration and transfer of survivors to fresh bottles every 2–3 days. UDIR3 indicates *UAS-DfhIR*. C96 indicates *C96* Gal4 driver. UAS-SOD1 indicates *UAS-Cu,Zn-superoxide dismutase*. UAS-SOD2 indicates *UAS-Mn-superoxide dismutase*. UAS-CAT indicates *UAS-catalase*. Note: Because CAT augmentation is confined essentially to the PNS, its activity is not detectable above background levels in whole-fly extracts (data not shown). However, in previous studies (17), ubiquitous expression of the same *UAS-Cat* transgene used here was shown to elevate CAT activity by nearly 5-fold.

extracts (data not shown). However, in previous studies (17), ubiquitous expression of the same *UAS-Cat* transgene used here elevated CAT activity by nearly 5-fold. As illustrated in Fig. 1a, the reduced life span of flies deficient in DFH is restored to control duration by expression of a *UAS-CAT* transgene. This result supports the proposed pathophysiological role of H_2O_2 and suggests that interventions that reduce this ROS have the potential to suppress the phenotypic outcomes of DFH deficiency.

Neither SOD1 nor SOD2 Restores Life Span. We then asked whether the life span of DFH-deficient flies could be restored by attenuating the availability of a different ROS, namely superoxide. However, expression of the superoxide scavengers, SOD1 or SOD2, is without effect (Fig. 1a). This result is important for two reasons. First, it provides evidence against the possibility that the “rescue” observed with *UAS-CAT* (above) is a technical artifact arising from the reduced expression of *UAS-DfhIR* by competition for a limited pool of Gal4 (As described in a subsequent section, this point is reinforced through the use of a UAS/Gal4-independent CAT transgene). Second, and more importantly, it suggests that unlike H_2O_2 , superoxide, whether in the cytoplasm

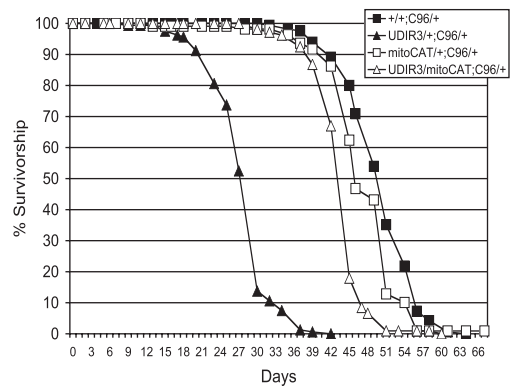


Fig. 2. mitoCAT rescues life span in DFH-deficient *Drosophila*. Note that mitoCAT does not extend life span in normal control adults. Survival of 125 males of each genotype on standard cornmeal food was followed at 25°C with enumeration and transfer of survivors to fresh bottles every 2–3 days. CAT activities in whole-fly extracts [normalized to +/-;C96/+ (100%)] were as follows: *mitoCAT*1/+;C96/+ = 340% \pm 20%; *mitoCAT*/UDIR3;C96/+ = 370% \pm 20%. Note that CAT activity is not impaired in flies deficient in DFH. This finding is important because frataxin is required for heme synthesis, and CAT requires heme for activity. UDIR3 indicates *UAS-DfhIR*. C96 indicates *C96*-Gal4 driver. mitoCAT indicates *mitochondrial catalase*.

(the domain of SOD1) or mitochondrial matrix (the domain of SOD2), is not directly involved in the life span-shortening effects of DFH deficiency.

As one of the controls for the above experiments, we examined the effects of overexpression of SOD1, SOD2, or CAT in DFH-sufficient flies by using the *C96*-Gal4 driver and were surprised to find that they all confer a modest but significant extension in life span compared with the control (Fig. 1b). However, when expressed in the context of DFH deficiency, only CAT restores the shortened life span phenotype. How DFH depletion suppresses the enhanced life span conferred by overexpression of SOD1 and SOD2 is not clear (see *Discussion*). The *UAS-SOD1*, *UAS-SOD2*, and *UAS-CAT* transgenes used in these studies all express their respective enzymatic activities at high levels in response to Gal4 induction (17).

Mitochondrial CAT Restores Life Span. That overexpression of the H_2O_2 -scavenging enzyme, CAT, restores the short life span of DFH-deficient *Drosophila* strongly implicates H_2O_2 as a critical element in the mechanism responsible for this early adult mortality phenotype. Although CAT provides robust restoration of life span in DFH-deficient flies, the disruption of iron metabolism arising from DFH deficiency should also occur in the mitochondrial matrix where DFH also resides (17, 32) and where mitochondrial superoxide emanating from the respiratory chain is reduced to H_2O_2 by SOD2 (33). In other words, all of the reactants for the generation of hydroxyl radical via Fenton chemistry should be present in the mitochondrial matrix of DFH-deficient flies, and enhanced scavenging of H_2O_2 in the mitochondrial matrix should equal or surpass that in the cytoplasm. However, unlike in mammals, CAT does not normally occur in the mitochondrial matrix of *Drosophila*. We therefore asked whether CAT ectopically expressed in the mitochondrial matrix would mitigate the life span-reducing effects of DFH deficiency. To determine the answer, we used a synthetic CAT transgene (*mitoCAT*) that targets CAT to the mitochondrial matrix via the 22-aa putative mitochondrial-targeting motif of the ornithine aminotransferase gene and that is expressed from the *Drosophila Cat* genomic promoter (34). This genomic promoter expresses in many adult tissues, including the adult PNS (35). As seen in Fig. 2, the *mitoCAT* transgene restores the life span of DFH-deficient flies to within 10% of controls. Thus,

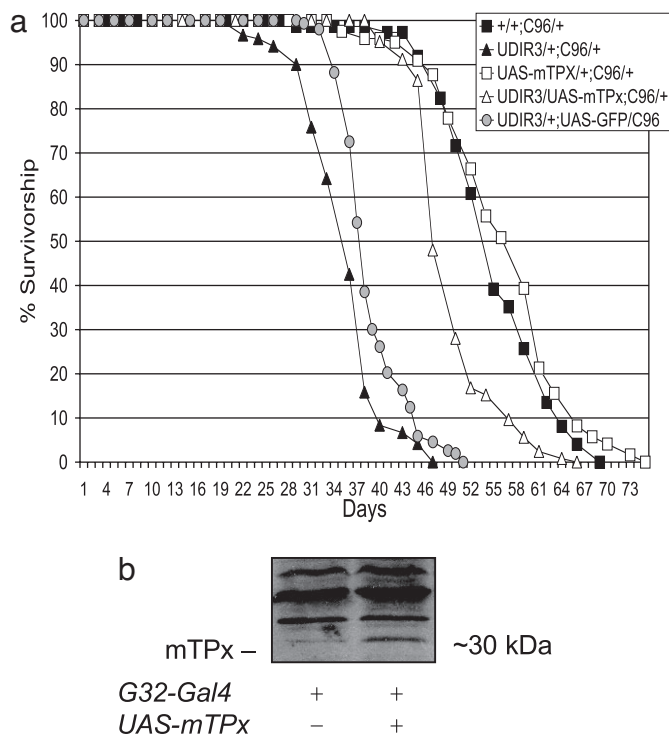


Fig. 3. Mitochondrial peroxiredoxin (mTPx) restores life span in DFH-deficient *Drosophila*. (a) Survival of 125 males of each genotype at 25°C with enumeration and transfer of survivors to fresh bottles every 2–3 days. Note that expression of *UAS-mTPx* does not extend life span of normal control adults. (b) Mitochondrial extracts from control (genotype +/+; *da*^{G32}/+) or adults (2–3 days old) expressing *UAS-mTPx* driven by the *da*^{G32}-Gal4 driver were electrophoresed, transferred to nitrocellulose membranes, and probed for mTPx with anti-TPx IgG. Approximate molecular masses are indicated. G32-Gal4 indicates *da*^{G32} driver. C96 indicates C96 Gal4 driver. UDIR3 indicates *UAS-DfhIR*. *UAS-mTPx* indicates *UAS-mitochondrial peroxiredoxin*. Note that in *b* the *da*^{G32} driver was used to demonstrate functionality of the *UAS-mTPx* transgene because, unlike C96, it provides the level and breadth of mTPx expression required for immunoblot detection.

CAT expressed ectopically in mitochondria can restore the life span of flies deficient in DFH nearly as well as cytoplasmic/peroxisomal CAT. Moreover, the rescue of DFH deficiency by the *mitoCAT* transgene, with its native genomic promoter, reduces potential concerns about the potential confounding effects of multiple UAS transgenes. Overall, the *mitoCAT* data provide further support for the conclusion that H₂O₂ is an important substrate in the pathology of DFH deficiency and that enzymatic scavenging of this oxidant in the biological context of DFH insufficiency can suppress the development of some of the associated deleterious phenotypes.

Mitochondrial Peroxiredoxin (mTPx) Restores Life Span. Among the reported biochemical consequences of DFH deficiency is the loss of iron homeostasis as reflected in the reduction in the level of the iron-binding protein, ferritin [FerHCH (17)]. Because CAT is a heme-containing protein, it is possible that the beneficial effects that accrue from the augmentation of CAT (or from the ectopic expression of *mitoCAT*) in DFH-deficient flies are not caused by the H₂O₂-scavenging activity of the enzyme but arise instead from the sequestering of potentially deleterious iron for the heme prosthetic groups required by the transgenic CAT. To examine this possibility, we overexpressed a H₂O₂-scavenging mTPx (36) in DFH-deficient flies. Because peroxiredoxins do not contain iron cofactors, restoration of life span by mTPx would argue against the possibility that supplemental CAT

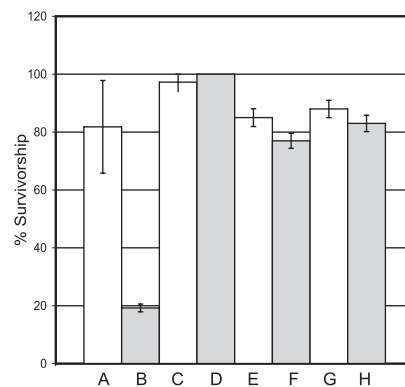


Fig. 4. DFH-deficient *Drosophila* are hypersensitive to H₂O₂ toxicity. Survival of males (7–9 days old) of each genotype on 1% sucrose containing 50 mM H₂O₂ was followed at 25°C with transfer of survivors to fresh vials every 12 h and final enumeration at 80 h. NB: +/+;C96/+ and *UDIR3*/+;C96/+ flies survive on 1% sucrose with no H₂O₂ beyond day 15 with little or no mortality. (A) +/+;C96/+, (B) *UDIR3*/+;C96/+, (C) *mitoCAT*/+;C96/+, (D) *UDIR3/mitoCAT*;C96/+, (E) +/+;UAS-mTPx/C96, (F) *UDIR3*/+;UAS-mTPx/C96, (G) *UAS-CAT*/+;C96/+, (H) *UDIR3/UAS-CAT*;C96/+. *mitoCAT* indicates mitochondrial catalase. *UAS-mTPx* indicates *UAS-mitochondrial peroxiredoxin*. *UAS-CAT* indicates *UAS-catalase*, C96 indicates the C96 Gal4 driver.

rescues by acting as a repository for reactive iron. Fig. 3 demonstrates that overexpression of *UAS-mTPx* restores the life span of the *DfhIR* flies to within 10% of controls. In support of the conclusion that H₂O₂ scavenging rather than iron sequestration underlies the observed rescue, we have also observed that chelation of dietary iron by bathophenanthrolinedisulfonic acid (37) does not improve life span of DFH-deficient flies (data not shown).

The issue raised earlier regarding possible confounding effects arising from the concurrent expression of multiple UAS transgenes can be seen in the data presented in Fig. 3a. As a control for this possibility, we expressed a control UAS transgene, *UAS-GFP*, along with *UAS-DfhIR*. In this experiment, the second transgene did exert a small positive impact on adult life span that likely arose not from the presence of GFP *per se* but from reduced expression of *UAS-DfhIR* through competition by *UAS-GFP* for Gal4. Although we believe that our interpretations of the effects of coexpression of *UAS-CAT* and *UAS-mTPx* with *UAS-DfhIR* are fundamentally correct, these data illustrate the potential for experimental artifacts with this system.

Adults Deficient in DFH Are Hypersensitive to H₂O₂. If endogenous H₂O₂ is an important factor in pathogenesis arising from DFH deficiency in *Drosophila*, we would predict that DFH-deficient flies would be hypersensitive to H₂O₂ exposure. Although susceptibility to H₂O₂ challenge typifies yeast and cell culture models of frataxin deficiency (3, 27–29), it has yet to be demonstrated in a whole-animal model of FRDA. Fig. 4 shows that DFH-deficient flies are indeed hypersensitive to H₂O₂ exposure. Moreover, this hypersensitivity can be alleviated by expression of the H₂O₂-scavenging enzymes CAT, mTPx, or *mitoCAT*.

CAT Restores mACON Activity. The aconitases are subject to reversible inactivation by ROS (38, 39). The vulnerability of the solvent-exposed Fe-S cluster of these enzymes to ROS attack provides a sensitive indicator of changes in ROS flux *in vivo* (38, 39). To determine further whether ectopically expressed CAT actually reduces the ROS burden in DFH-deficient flies, we used the activities of mACON and cytoplasmic aconitase (cACON) to assess the level of ROS flux in DFH-deficient flies expressing *mitoCAT*. To allow a better visualization of the impact of

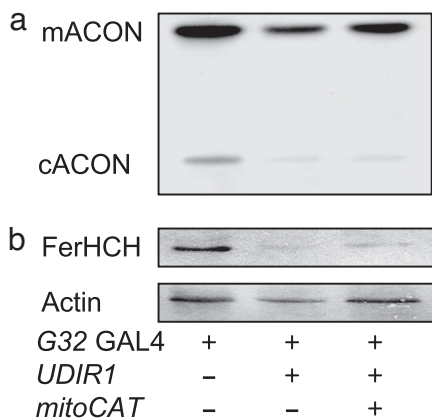


Fig. 5. mitoCAT restores the activity of mACON but not cACON in DFH-deficient *Drosophila*. (a) ACON activities in extracts of 2- to 3-day-old adult males were assayed after electrophoretic separation. (b) Total protein extracts of 2- to 3-day-old adult males were electrophoresed, transferred to nitrocellulose membranes, probed with anti-FerHCH antibodies, and then reprobed with anti-actin antibodies as a loading control. G32 GAL4 indicates *daG32-Gal4* driver; *mitoCAT* indicates the *mitoCat* transgene; FerHCH indicates ferritin heavy-chain homolog. mACON activities normalized to the G32/+ control (100%) are: UDIR1/G32, $43 \pm 2\%$; *mitoCat*/+;UDIR1/G32, $66 \pm 2\%$. cACON activities normalized to the G32/+ control (100%) are: UDIR1/G32, $25 \pm 6\%$; *mitoCat*/+;UDIR1/G32, $28 \pm 5\%$. ACON activities were compiled from densitometric scans of three separate experiments

mitoCAT on aconitase activity, we used flies carrying the *daG32-Gal4* driver (Flybase: daG32 Gal4) because it provides more widespread expression of UAS transgenes than the *C96-Gal4* driver, thereby exposing a greater proportion of the total aconitases in the fly to inactivation by the conditions imposed by *DfhIR*. In addition, the *mitoCAT* transgene was used to target the H₂O₂ scavenger to the mitochondrial matrix where mACON resides and, as above, to avoid possible Gal4 dilution artifacts that might occur in combination with the *UAS-DfhIR* transgene. As shown in Fig. 5a, the expression of mitoCAT restores mACON activity in DFH-deficient flies. In contrast, cACON (Fig. 5a) and the iron response protein/FerHCH couplet (Fig. 5b), both of which are constituents of the cytoplasmic compartment, remain unaffected.

Discussion

Deficiency of the mitochondrial iron chaperone, frataxin, is the primary molecular lesion in FRDA. Discovering how this deficiency leads to the clinical outcomes of the disease is urgent. Understanding the etiology of the disease has been aided by two salient findings, namely, that frataxin deficiency leads in turn to impaired intracellular iron homeostasis (7, 17, 40–42) and that oxidative stress plays a role as either a primary or secondary effector of FRDA pathology (for review, see refs. 25 and 26). At the outset of the present work, these and other findings had raised the possibility that at least part of the pathophysiology of FRDA arises from ROS (unspecified) emanating from impaired Fe-S-containing components of the respiratory chain. This work narrows the paradigm of ROS-mediated FRDA pathology with evidence from a whole-animal model that H₂O₂, specifically, is an important substrate in the pathology of frataxin deficiency, and perhaps most importantly, is an efficacious target for alleviating the deleterious effects of frataxin deficiency.

In an earlier study with our *Drosophila* FRDA model (17), we showed that widespread RNAi-mediated DFH deficiency with the ubiquitous Gal4 driver, *daG32*, caused delayed metamorphosis, impaired eclosion, and severe early mortality of the few surviving adults, and that overexpression of SOD1, SOD2, or

CAT by the *daG32-Gal4* driver did not improve the capacity of DFH-deficient larvae to develop into adults. In the present work, we have focused DFH deficiency on the adult PNS, which spares preadult effects, leaving reduced adult life span as the predominant phenotype. Using this system, we now report that overexpression of the H₂O₂-scavenging enzymes CAT, mitoCAT, or mTPx rescues the shortened life span brought about by DFH deficiency (Figs. 1–3). Each of these enzymes is known to be cytoprotective against H₂O₂ when overexpressed in either whole flies or in *Drosophila* cells in culture (31, 36, 43). Furthermore, although all of these enzymes reduce H₂O₂ to water, mTPx overexpression was not as effective as CAT or mitoCAT in rescuing the life span of DFH-deficient *Drosophila*. Differential levels of expression of the enzymes could underlie this difference, or the differential rescue could be caused by the reliance of mTPx on a secondary substrate, reduced thioredoxin, not required by either CAT or mitoCAT. Further to this point, we should add a cautionary note against comparing the relative “efficiencies” of rescue by the three enzymes. The domain of expression of these transgenes was very different: mitoCAT and mTPx were expressed ubiquitously, whereas cytoplasmic CAT was confined to the PNS, and we do not know what the relative expression of mitoCAT and mTPx actually was in the PNS.

Two ancillary observations merit comment. First, augmentation of SOD1, SOD2, or CAT provides a modest extension of life span in DFH-sufficient adults (Fig. 1b); however, of these three, only augmentation with CAT (and mitoCAT and mTPx) rescues the early mortality brought about by DFH deficiency. Second, the extended life span provided by augmentation with SOD1 or SOD2 (but not by CAT) in DFH-sufficient adults is suppressed in DFH-deficient adults (Fig. 1a). We propose that these apparently disparate observations share a common mechanistic origin. Resistance of DFH-deficient adults to rescue by augmentation with SOD1 or SOD2 argues that superoxide is not a component of the pathophysiological mechanism of DFH deficiency, which means that although DFH deficiency may suppress overall respiratory chain activity through the impaired synthesis and restoration of essential Fe-S clusters (17, 32), such suppressed respiratory chain activity is likely not accompanied by an increased flux of superoxide. If it were, augmentation with SOD2 and/or SOD1 would have restored life span of DFH-deficient adults. By this reasoning, the life span extension of DFH-sufficient adults by augmentation with SOD1 or SOD2 would appear to require a normally functioning respiratory chain. In this context, it might be informative in future experiments to examine the impact of SOD1 or SOD2 augmentation on cytoplasmic and mitochondrial aconitase activities in DFH-deficient flies. In keeping with our observations, it has been reported that increased expression of SOD1 and exposure to a small-molecule SOD2 mimetic does not improve murine FRDA cardiomyopathy (44).

It has recently been demonstrated that FRDA patient lymphoblasts exhibit increased generation of H₂O₂ (45). Although it is uncertain whether the deleterious phenotypes arising in the *Drosophila* model of FRDA are caused by increased levels of H₂O₂, increased susceptibility to H₂O₂-mediated damage, or a combination of these factors, it is clear that increasing H₂O₂-scavenging capacity improves pathology (as measured by life span). It is possible that the observed improvement in pathology is due at least in part to increased cellular energy production. The observed restoration of mACON activity in *DfhIR* flies expressing mitoCAT implies that the loss of mACON activity (and perhaps loss of mitochondrial respiratory complex activity) reported in ref. 17 could be at least partially caused by H₂O₂-mediated oxidative damage to iron cofactors. The exquisite sensitivity of the mACON Fe-S cluster to frataxin deficiency has been well established (17, 23, 32, 46). Thus, the increased activity of ACON by H₂O₂ scavenging may foreshadow discovery of pro-

tection of other iron cofactor enzyme activities important in electron transport and oxidative phosphorylation and improve the noted decrease in ATP production associated with FRDA (47).

Although H₂O₂ scavenging improved pathology, there were aspects of the phenotype not improved by this intervention. cACON activity levels and FerHCH levels were unaffected by H₂O₂-scavenging capacity, indicating that intracellular iron homeostasis was not impacted (Fig. 5*b*). These data underscore the complex nature of frataxin deficiency phenotypes and likely underlie the inability of increased mTPx and mitoCAT levels to correct for DFH deficiency completely (Figs. 2 and 3). Furthermore, these data underscore the need for investigation of combinatorial therapies addressing the many symptoms associated with FRDA.

FRDA manifests its most debilitating effects in the PNS and the heart. For this reason, we chose to use the C96-Gal4 driver as a central element of the *Drosophila* model of FRDA. Although C96, like most Gal4 drivers, does not exhibit strict tissue specificity, the results of its use in this work are nominally consistent with the notion that, as in mammals, the PNS of *Drosophila* is particularly susceptible to frataxin deficiency. To test further the equivalence of tissues sensitive to frataxin deficiency in mammals and *Drosophila*, we recently examined the consequences of RNAi-mediated *Dfh* deficiency by using Gal4 drivers that focus expression in several other tissues, including the motor neurons, skeletal muscle, and the heart. Of these tissues, only frataxin deficiency in the heart has thus far proved to be deleterious in the *Drosophila* model (P.R.A. and J.P.P., unpublished data). If confirmed, it will be of interest from a therapeutic perspective to determine whether the symptoms arising from cardiac-focused DFH deficiency can be also mitigated by H₂O₂ scavenging.

Our results underscore the importance of discriminating between specific ROS involved in disease pathology. Several potential therapeutics for FRDA are in advanced clinical trials (48). Among the most promising are CoQ10 (ubiquinone) and the parabenzoquinone derivatives idebenone and MitoQ, which are designed to scavenge or reduce escape of electrons from complex I and complex II of the electron transport chain, which leads in turn to the formation of ROS. Our results support continued development of ROS-based therapeutics and suggest that a new focus on H₂O₂ may be clinically rewarding.

Materials and Methods

Drosophila Stocks and Culture Methods. *Drosophila* stocks were maintained at 25°C on standard cornmeal agar medium unless otherwise stated. CO₂ was used to anesthetize adult flies throughout, allowing at least 5 h of recovery at room temperature before the onset of experimentation to avoid potential latent effects of the anesthesia (49). The broadly expressing *da^{G32}*-Gal4 driver (Flybase: daG32 Gal4) and the C96-Gal4 (50) driver (which promotes robust expression in the PNS on a background of widespread, low-level expression) were originally obtained from G. Boulianne (University of Toronto, Toronto, ON). *UAS-Sod1*, *UAS-Cat*, *UAS-Sod2*, and *UAS-Dfh Inverted-Repeat UDIR1* flies were developed in this laboratory as described in ref. 17; *UAS-mTPx* was

generated in the Orr laboratory using similar methods. Expression of *UDIR3* leads to phenotypes as severe, or more severe, than those reported for *UDIR1* (17). *UAS-GFP* flies were obtained from the Bloomington Stock Center. To avoid potential recessive effects, Gal4 drivers, UAS constructs, and the *mitoCAT* transgene were used in hemizygous configuration for all experiments. Genetic backgrounds containing the C96-Gal4 driver, *UDIR3*, and either a second UAS transgene or *mitoCAT* were obtained by first generating *UDIR3/SM5;C96/TM3* males that were then crossed to the appropriate UAS transgene or *mitoCAT* females. Controls for these genetic backgrounds were generated by substituting the second chromosome of the *UDIR3 w¹* progenitor stock for that containing *UDIR3* in the above crossing scheme. The genetic backgrounds containing the *da^{G32}*-Gal4 driver, *UDIR1*, and *mitoCAT* were obtained by first generating *mitoCAT/SM5;da^{G32}/TM3* males that were subsequently crossed to *UDIR1* females. Controls for this genetic background were generated by substituting the second chromosome of the *mitoCAT* progenitor stock for that containing *mitoCAT* in the above crossing scheme. *mitoCAT/SM5;da^{G32}/TM3* and *UDIR3/SM5;C96/TM3* stocks were expanded and briefly maintained by crossing males of these genotypes to *Xa/SM5;TM3*.

Western Immunoblotting. Preparation of *Drosophila* samples for Western immunoblotting was performed as described in ref. 51. For this and all other procedures, protein levels were determined with the Bio-Rad protein assay. Samples were separated on 4% stacking, 15% separating SDS/polyacrylamide gels. The protein was transferred to Hybond-ECL nitrocellulose membrane (Amersham Pharmacia), blocked with 3% gelatin, and probed with appropriate primary antibody and secondary antibody conjugates. Rabbit polyclonal antibodies against the *Drosophila* ferritin heavy-chain homolog (a gift from F. Missirlis), or peroxiredoxin DPX-4783 (from W.C.O., described in ref. 36) were used in combination with goat anti-rabbit IgG horseradish peroxidase conjugate (Stressgen) and detected with ECL Western blotting detection reagents (Amersham Pharmacia). Mouse anti-actin monoclonal IgM antibodies (Developmental Studies Hybridoma Bank) were used in combination with anti-mouse IgM alkaline phosphatase conjugate and detected according to the manufacturer's instructions (Sigma). Prestained protein ladders (Invitrogen) were run in parallel with samples to estimate protein molecular weight.

Catalase Assay. Catalase activity was determined spectrophotometrically by following H₂O₂ breakdown at 230 nm as described in ref. 52.

ACON Assay. Mitochondrial and cytoplasmic ACON activities in whole-animal extracts were assayed jointly after electrophoretic separation (51).

Life Span Determinations. Survival of 125–150 adult males on standard cornmeal medium was followed at 25°C with transfer of survivors to fresh bottles every 2–3 days. The life span data shown are representative of three to five independent replicates.

H₂O₂ Toxicity. Survival of 7- to 9-day-old males in groups of 10 was assessed on 1% sucrose or 1% sucrose fortified with 50 mM H₂O₂ at 25°C. Sucrose solution (450 μl) was absorbed into eight layers of 1.5-cm² squares of KimWipe placed in standard fly vials. Vials were changed every 48 h with enumeration between 80 and 90 h.

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