Cell Metabolism
Article

Expression of the *Ciona intestinalis* Alternative Oxidase (AOX) in *Drosophila* Complements Defects in Mitochondrial Oxidative Phosphorylation

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DOI 10.1016/j.cmet.2009.03.004

SUMMARY

Defects in mitochondrial OXPHOS are associated with diverse and mostly intractable human disorders. The single-subunit alternative oxidase (AOX) found in many eukaryotes, but not in arthropods or vertebrates, offers a potential bypass of the OXPHOS cytochrome chain under conditions of pathological OXPHOS inhibition. We have engineered *Ciona intestinalis* AOX for conditional expression in *Drosophila melanogaster.* Ubiquitous AOX expression produced no detrimental phenotype in wild-type flies. However, mitochondrial suspensions from AOX-expressing flies exhibited a significant cyanide-resistant substrate oxidation, and the flies were partially resistant to both cyanide and antimycin. AOX expression was able to complement the semilethality of partial knockdown of both *cyclope* (COXVIc) and the complex IV assembly factor *Surf1*. It also rescued the locomotor defect and excess mitochondrial ROS production of flies mutated in *dj-1*β, a *Drosophila* homolog of the human Parkinson's disease gene *DJ1*. AOX appears to offer promise as a wide-spectrum therapeutic tool in OXPHOS disorders.

INTRODUCTION

Dysfunction of the mitochondrial oxidative phosphorylation (OXPHOS) system is a recognized feature of many disease states. These range from the classical mitochondrial encephalomyopathies, both pediatric (Moslemi and Darin, 2007) and adult-onset (Finsterer, 2004), to cases of common multifactorial disorders such as diabetes (Maassen et al., 2004; Kim et al., 2008) and deafness (Kokotas et al., 2007). In addition, many late-onset neurodegenerative disorders, such as Parkinson's disease, are associated with OXPHOS dysfunction, which has been postulated as instrumental in pathogenesis (Dimauro and Schon, 2008; Reeve et al., 2008). Mostly these disorders are progressive and incurable.

The OXPHOS system (Smeitink et al., 2001) comprises the five multisubunit complexes involved in respiratory electron flow and ATP synthesis, plus the entire apparatus for their biosynthesis. This includes the machinery of mtDNA maintenance and gene expression, four of the OXPHOS complexes (I, III, IV, and V) containing mtDNA-encoded subunits. Complex II, plus several other non-proton-pumping dehydrogenases, feed electrons directly into the downstream (cytochrome) portion of the respiratory chain, complexes III and IV.

OXPHOS disease can result from deficiency or dysfunction of any component of this system. Genetic targets include both structural subunits of the OXPHOS complexes, plus those involved in biosynthesis and assembly (Smeitink et al., 2001): in total, over 200 different gene products. OXPHOS inhibition can also result from the action of toxins that target specific enzymatic steps, such as complexes I (rotenone), III (antimycin), IV (cyanide), or V (oligomycin).

OXPHOS dysfunction entrains a range of metabolic disturbances in addition to bioenergy deficit, and these may underlie much of the pathology of OXPHOS disease (Smeitink et al., 2006). Inhibition of electron flow can result in the excess production of reactive oxygen species (ROS) at complexes I and/or III if the quinone pool becomes overreduced. Excessive ROS production can damage lipids, proteins, carbohydrates, and nucleic acids, and if such damage is not repaired, it can result in cell death. The need to recruit cytosolic shunts for the reoxidation of NADH, notably lactate dehydrogenase, results in the production of lactic acid, which leads to cellular and systemic acidosis. Finally, limitations on NADH reoxidation as well as on substrate flux may result in a more general impairment of metabolism, including deficiency of carbon skeletons for biosynthesis and deranged calcium homeostasis.

Most eukaryotes possess an alternative mitochondrial respiratory chain, which can bypass the OXPHOS system under specific physiological conditions. Plants, in particular, typically

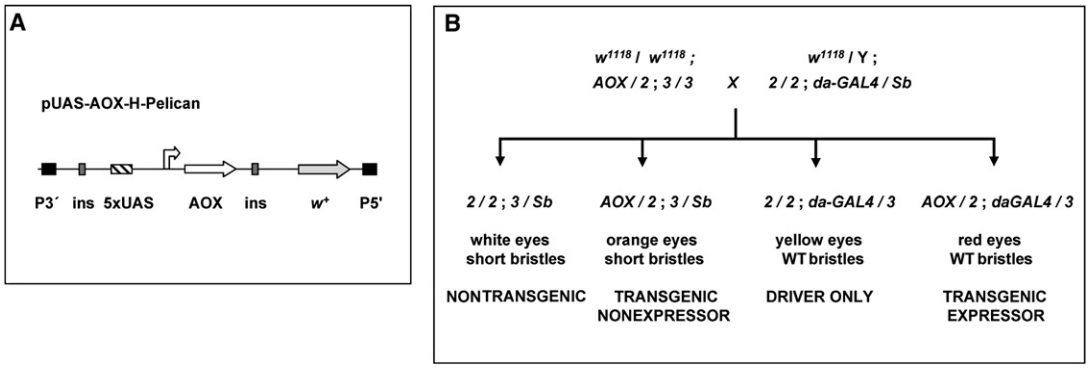

Figure 1. Scheme for AOX Expression in *Drosophila*

(A) Schematic map of transgenic construct pUAS-AOX-H-Pelican. For construction details, see Figure S1. The 3′ and 5′ P-element ends (P3′, P5′, black) flank the two coding regions, i.e., of the selectable eye-color marker w^+ (light gray) under its own promoter and AOX under the control of the GAL4-dependent 5×UAS element (cross-hatched) combined with the (reiterated) weak basal promoter of the Hsp70 gene (block arrow). The AOX expression cassette is flanked by gypsy insulator elements (ins, dark gray).

(B) Crossing scheme to obtain AOX-expressing progeny. Transgenic hemizygotes (AOX transgene on chromosome 2, carrying the mini-white w^+ eye-color marker; *2* denotes wild-type chromosome 2) were crossed to hemizygotes for the ubiquitous *da-GAL4* driver (on chromosome 3, balanced against the *Sb* marker; *3* denotes wild-type chromosome 3). All flies were in the w^{1118} genetic background. Different progeny classes were identified on the basis of eye-color and bristle morphology phenotypes. Nontransgenic, nonexpresser, and "driver-only" progeny represent controls for the various experiments. An analogous strategy was used for transgenes on chromosome 3, using the ubiquitous *Act5C-GAL4* driver on chromosome 2 balanced against *CyO*. For all AOX transgenic lines tested, the four progeny classes eclosed in approximately equal numbers. Where not indicated in subsequent figures, AOX expressers and nonexpressers were derived from AOX transgenic line F6 using this crossing scheme.

have several NADH dehydrogenases that bypass complex I (Rasmusson et al., 2004), plus a mitochondrially localized alternative oxidase (AOX), which bypasses the cytochrome chain, passing electrons directly from ubiquinol to molecular oxygen (Juszczuk and Rychter, 2003). AOX is an integral nonheme iron protein of the inner mitochondrial membrane, believed to function as a homodimer. It is thought to be essential for maintaining mitochondrial metabolic flux and redox homeostasis when ATP levels are high, such as in plants under daylight conditions (Rustin and Lance, 1986; Noguchi and Yoshida, 2008). In fungi, AOX has been implicated in the control of longevity and resistance to oxidative stress (Lorin et al., 2001). AOX is also found in many metazoans, including annelids, mollusks, and urochordates (McDonald and Vanlerberghe, 2004, 2006), although not in arthropods or vertebrates, and it has been suggested to provide resistance to oxidative stress (Abele et al., 2007).

We reasoned that an AOX bypass might alleviate many of the pathological consequences of OXPHOS inhibition. Since AOX is absent from humans, we tested this idea by introducing to human cells an AOX cDNA from the urochordate *Ciona intestinalis*, i.e., a representative of a sister taxon to the vertebrates. Using an inducible expression system, we demonstrated (Hakkaart et al., 2006) that *Ciona* AOX is mitochondrially targeted in human cells, confers cyanide-resistant respiration, and protects against metabolic acidosis, oxidative stress, and cell death when cells are treated with OXPHOS inhibitors such as antimycin or cyanide.

Here, we tested whether AOX expression can be tolerated in the whole organism and whether it can alleviate the effects of OXPHOS dysfunction in vivo using *Drosophila*, a credible and tractable animal model for many mitochondrial and neurodegenerative pathologies (Sánchez-Martínez et al., 2006; Marsh and Thompson, 2006). We report that ubiquitous expression of *Ciona* AOX in the fly is benign, enabling cyanide-resistant substrate oxidation in vitro and a substantial resistance to OXPHOS toxins in vivo. In addition, AOX partially compensated the mutant phenotypes of several disease-equivalent mutations.

RESULTS

AOX-Expressing Flies Are Viable and Healthy

To create lines of *Drosophila* transgenic for AOX, we recloned the *Ciona* AOX cDNA in a modified P-element vector under the control of a GAL4-dependent promoter and protected by insulator elements (Figure 1A). Standard microinjection was used to create a set of transgenic lines with different conditionally expressible AOX insertions. To test whether constitutive AOX expression was compatible with life in *Drosophila*, we crossed transgenic lines hemizygous for GAL4-dependent AOX with lines carrying the ubiquitously expressing *da-GAL4* driver, using the scheme of Figure 1B. Flies of all four phenotypic classes eclosed in approximately equal numbers, using each of three different AOX transgenic lines (F6, F17, and F24). We confirmed *AOX* transgene expression at the RNA level by means of in situ hybridization (Figure 2A) and quantitative RT-PCR (Figures 2B and 2C) and at the protein level by western blotting (Figures 2D and S1B) and whole-mount immunocytochemistry of larvae (Figure S1C).

AOX transgene expression was generally higher in males than females (Figures 2B and 2C), and the expressed protein was highly enriched in mitochondria (Figures 2D and S1C). GAL4-dependent AOX expression was 300–1000 times greater than without induction and was comparable with that of the highly expressed reference gene *RpL32* (or GAPDH), used as an internal standard in all assays. Similar expression levels were obtained when driven by either of two ubiquitous GAL4 drivers (*da-GAL4* or *Act5C-GAL4*), and graded expression at larval stage L3 was obtained using the drug-inducible *tub-GS* driver and different concentrations of the inducing drug RU486.

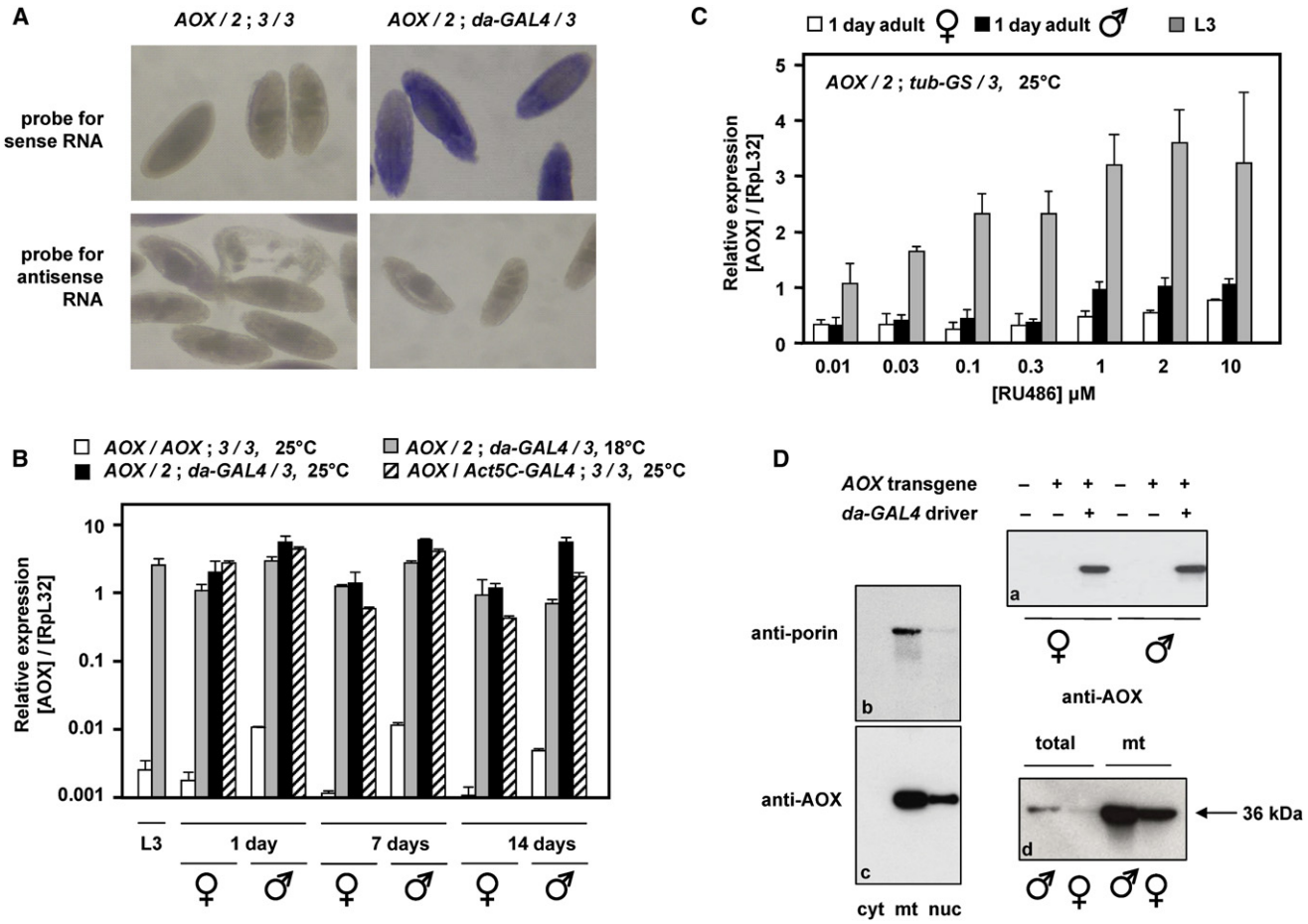

Figure 2. AOX Can Be Ubiquitously Expressed in *Drosophila*

(A) Whole-mount in situ hybridization to AOX-expressing and nonexpressing embryos, probed for AOX sense and antisense transcripts as indicated ("sense" probe means labeled RNA of the antisense strand and vice versa). The different classes of embryos were obtained by crossing homozygous AOX transgenic females either with wild-type males or with homozygous *da-GAL4* driver males.

(B and C) Q-RT-PCR of AOX mRNA relative to *RpL32* mRNA, from hemizygous AOX transgenic flies (line F6, L3 larvae, adult males and females of different ages as shown) combined with different GAL4 drivers and growth temperatures or with the *tub-GS* driver in presence of different concentrations of RU486 throughout development. Genotypes are indicated here and in other figures as follows. *AOX* denotes presence of a *P*{*UAS-AOX*} transgene from line F6, if no subscript indicates otherwise. Numbers *2* and *3* denote the corresponding wild-type chromosomes. GAL4 driver transgenes are denoted as *da-GAL4*, etc. Wild-type flies are w^{1118}, the genetic background used for all the transgenic lines. AOX RNA was undetectable by Q-RT-PCR in lines not carrying the AOX transgene. Means are ± SD.

(D) Western blot of protein extracts (30 µg) from subcellular fractions and flies of sex and genotype indicated, probed with anti-AOX and anti-porin antibodies. Subpanel a: mitochondrial protein extracts from AOX-expressing flies and nonexpressing controls. Subpanels b and c: cytosolic (cyt), mitochondrial (mt), and crude nuclear (nuc) fractions. Subpanel d: total and mitochondrial extracts, all from AOX-expressing (AOX^{F6} / *2* ; *da-GAL4* / *3*) flies.

Ubiquitously AOX-expressing flies exhibited a very slight (⩽0.5 days) but statistically significant developmental delay (Figure 3A), although this may be due solely to the burden of transgene expression, since flies expressing GFP under the same conditions also showed a slight delay.

AOX-expressing adults of both sexes were fertile and, when mated to wild-type flies, produced healthy offspring in normal numbers. AOX-expressing flies of both sexes showed a slightly exaggerated weight loss as young adults compared with nonexpressers or with flies expressing GFP (Figure 3B).

AOX Supports KCN-Resistant Substrate Oxidation

To test whether AOX expression in *Drosophila* can contribute to electron flow and thus bypass complexes III and IV, we measured oxygen consumption of suspensions of *Drosophila* mitochondria in the presence of various substrate/inhibitor combinations. In preliminary experiments, mitochondria from AOX-expressing progeny of three separate AOX transgenic lines supported a substantial cyanide-resistant substrate oxidation (up to 50% of uninhibited levels) when supplied with pyruvate plus succinate (data not shown). To minimize background effects, we backcrossed the AOX transgene from the three transgenic lines, as well as the *da-GAL4* driver, over six generations into a reference wild-type background (Dahomey w^{-}) and analyzed mitochondrial substrate oxidation using a standard pyruvate-plus-proline substrate cocktail (Figures 4 and S2). The respiratory control index was unchanged by AOX expression. However, after cyanide addition, mitochondria

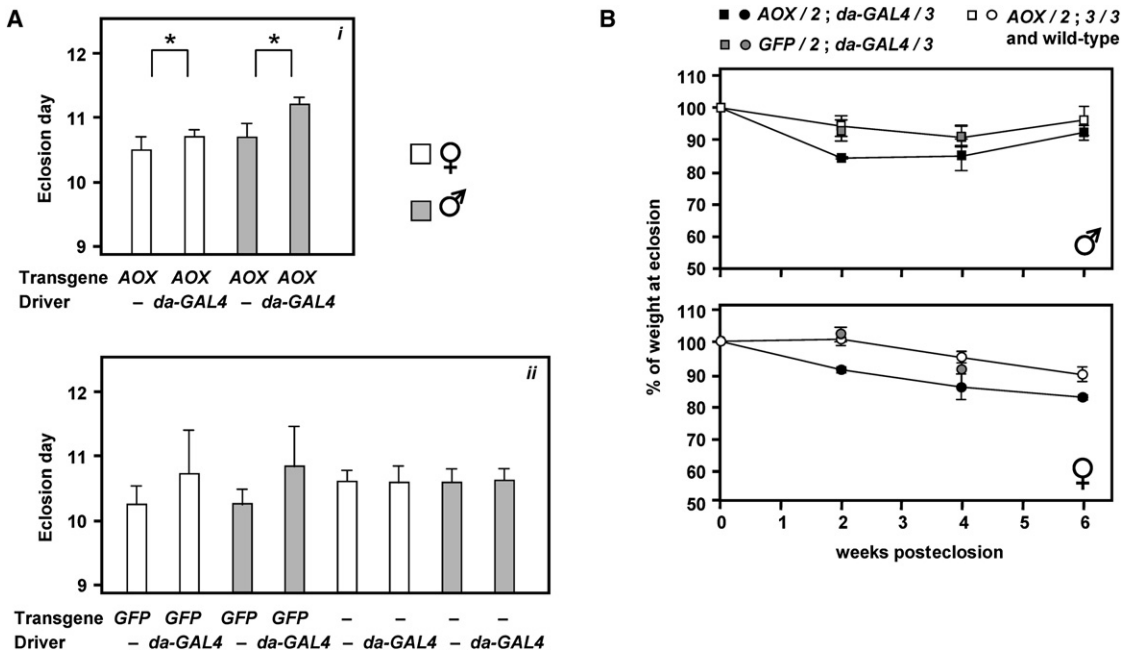

Figure 3. AOX Expression in *Drosophila* Is Benign

(A) Eclosion day of flies of the sex and genotype shown. Means ± SD analyzed by one-way ANOVA (Holm-Sidak); $p < 0.001$ for pairwise comparisons as indicated by asterisk. *GFP* denotes the presence of a *P{UAS-GFP}* transgene, on chromosome 2 in this experiment and in other figures, except where indicated. Flies were progeny from wild-type, homozygous AOX^{F6}, or *GFP* control transgenic females mated either with wild-type males (respectively giving wild-type, *AOX*, and *GFP*-nonexpressing progeny) or with homozygous *da-GAL4* driver males (giving *AOX* and *GFP* expressers and "driver-only" controls). The developmental delay produced by GFP expression was similar to that produced by AOX, although in this case not statistically significant ($p = 0.42$).

(B) Weight loss profiles (percent wet weight at eclosion) in young adult flies of the sex and genotype shown, obtained as in (A). *GFP*-nonexpressing flies (*GFP / 2*; *3 / 3*) gave weight loss profiles indistinguishable from controls, but for clarity, these are not shown in the figure. Means are ± SD.

from AOX-expressing flies typically maintained 10%–20% of the uninhibited state 3 oxygen consumption. Subsequent addition of propyl gallate (data not shown) or salicylhydroxamic acid (SHAM) (Figure 4A), two well-characterized inhibitors of alternative oxidases, abolished the residual cyanide-resistant oxidation. However, if SHAM was added first, it had no effect (Figure S2H), indicating that AOX did not contribute appreciably to electron flow under uninhibited conditions. In one transgenic line analyzed in greater detail (F6), the proportion of cyanide-resistant oxidation was increased when measured at 29°C (Figure 4C). In aging flies (at 50% of mean life span), where state 3 substrate oxidation was diminished, the cyanide-resistant proportion was maintained (Figure 4C). On sn-glycerol-3-phosphate, a substrate that feeds electrons directly to complex III, AOX-expressing flies also manifested cyanide-resistant oxidation (Figure S2E). No cyanide-resistant substrate oxidation could be detected in mitochondrial suspensions from flies carrying the *da-GAL4* driver but not *AOX*, nor those expressing a control transgene, GFP (Figures 4C and S2F). OXPHOS enzyme activities in AOX-expressing flies were indistinguishable from those of nonexpressers (Figure 4D), including cytochrome *c* oxidase (complex IV). Note that this is expected, since AOX is a ubiquinol oxidase, not a cytochrome *c* oxidase.

AOX Confers OXPHOS Toxin Resistance In Vivo

To determine whether AOX can function in vivo to bypass the cytochrome chain under OXPHOS stress conditions, we tested flies for resistance to cyanide and antimycin: inhibitors, respectively, of complexes IV and III. Because of the volatility of HCN, we established an assay to test the short-term toxicity of cyanide to adult flies by placing them inside plugged vials containing agarose impregnated with 10 mM KCN. Control flies were incapacitated within 5 min of such treatment, whereas AOX-expressing flies remained active during 20–30 min of cyanide exposure (Figure 5A). All of the AOX-expressing flies exposed to KCN recovered from paralysis overnight, whereas only 1%–2% of nonexpressing flies did so. Resistance to antimycin was tested by addition of the drug at various concentrations to fly food. Control eggs laid on 30 μg/ml antimycin failed to develop, and those laid on 10 μg/ml antimycin developed only to first instar (L1) larvae, whereas AOX-expresser eggs laid on antimycin-containing medium developed to adults in substantial numbers (Figures 5B and S2J), albeit with a considerable developmental delay (3–5 days at 25°C). Adult AOX-expressing flies were also resistant to antimycin at 30 or 100 μg/ml, and some even survived for 3 days at the highest concentration (Figure 5C). Control flies all succumbed to the toxin, even at the lower dose, within 2 days.

AOX Complements Partial Knockdown of COX

In *Drosophila*, complete functional loss of cytochrome oxidase (COX), e.g., via homozygosity for a null mutation of *cyclope* encoding the COXVIc structural subunit of complex IV, is lethal.

AOX expression was unable to complement this complete loss of function (data not shown). However, it did rescue the lethality

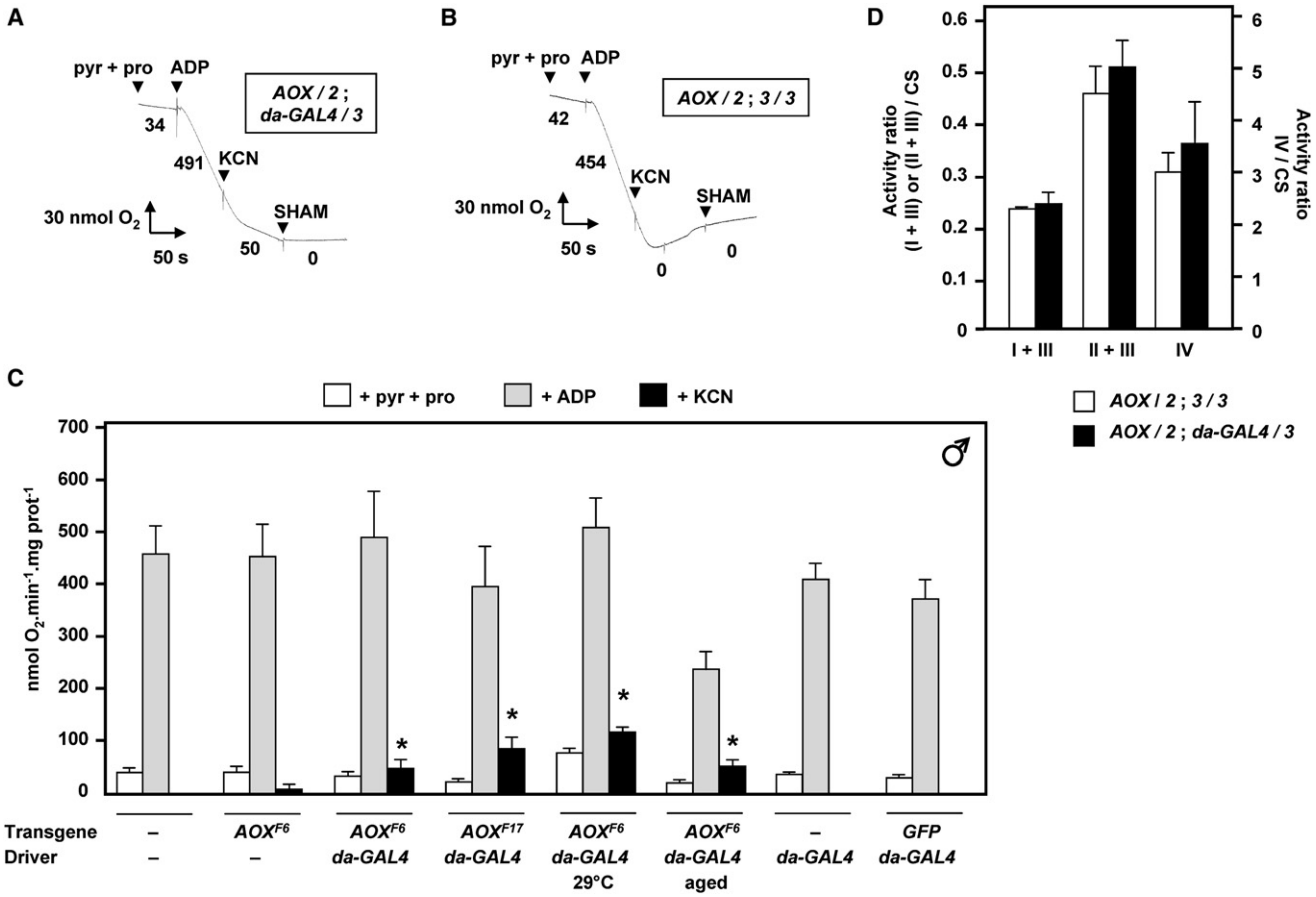

Figure 4. Mitochondria from AOX-Expressing Flies Exhibit Cyanide-Resistant Substrate Oxidation

(A–D) Representative polarographic traces showing rates of oxygen consumption (A and B) plus compiled polarographic data (C) and respiratory chain complex activities (D) from flies of the sex, age, growth/assay temperature, and genotype indicated. Genotypes abbreviated as elsewhere; transgenes in all cases hemizygous. The traces shown in (A) and (B) are for males of transgenic line F6, with or without the *da-GAL4* driver. The data of (C), in each case mean values ± SEM of at least four independent measurements, are abstracted from those of Figure S2. Substrate mix was 5 mM pyruvate plus 5 mM proline (pyr + pro) in all cases shown. Asterisks indicate data classes significantly different from corresponding wild-type values (ANOVA, $p < 0.001$). The data in (D) are from flies derived as in Figure 2, in each case showing mean values ± SD of at least four independent measurements, normalized against citrate synthase (CS) activity.

caused by partial knockdown of COX, effected using RNAi technology against either *cyclope* or the complex IV assembly factor *Surf1*. We combined GAL4-dependent dsRNA lines for each of these genes with GAL4-dependent AOX, plus a suitable driver, thus simultaneously knocking down *cyclope* and expressing AOX. Using the ubiquitous *da-GAL4* driver, AOX was able to rescue the semilethality of *cyclope* knockdown (Figures 6A, 6B, and S3A–S3E). The clearest result was obtained by culturing the flies at 18°C, combined with a 2 day heat shock at 30°C during larval stage L3 (Figure 6A), conditions under which very few flies knocked down for *cyclope* eclosed, unless AOX was coexpressed. At 25°C (Figures S3A and S3E), AOX abrogated the developmental delay and small size of the eclosing progeny knocked down for *cyclope*. Expression of an inert transgene, *GFP*, failed to effect rescue (Figure S3C). Mitochondrial COX activity from AOX-expressing flies knocked down for *cyclope* was less than half that from AOX-expressing control flies (Figures 6B and S3D). In contrast, AOX expression by the same strategy was unable to complement the lethality of knockdown of *CG3731*, which encodes a protein with a dual function as a subunit of OXPHOS complex III and subunit β of the mitochondrial matrix processing peptidase. The rescue of *cyclope* knockdown by AOX is thus, by all these criteria, specific.

In the case of *Surf1* knockdown (Zordan et al., 2006), we were able to demonstrate rescue using the inducible *tub-GS* driver in combination with different doses of the inducing drug RU486 (Figures 6C and 6D). At the highest doses, AOX was unable to rescue lethality, whereas at low doses, knockdown was insufficient to produce complete lethality, even in control flies. However, at intermediate doses, where *Surf1* knockdown alone was still lethal or semilethal, concomitant AOX expression under the control of the same driver rescued the lethality. The extent of *Surf1* mRNA knockdown, as measured by Q-RT-PCR, was similar in AOX-expressing and nonexpressing flies cultured at the discriminating drug concentration of 0.2 μM (Figure S3F). Importantly, expression of an inert transgene, GFP, under identical conditions failed to rescue the lethality (Figure 6D).

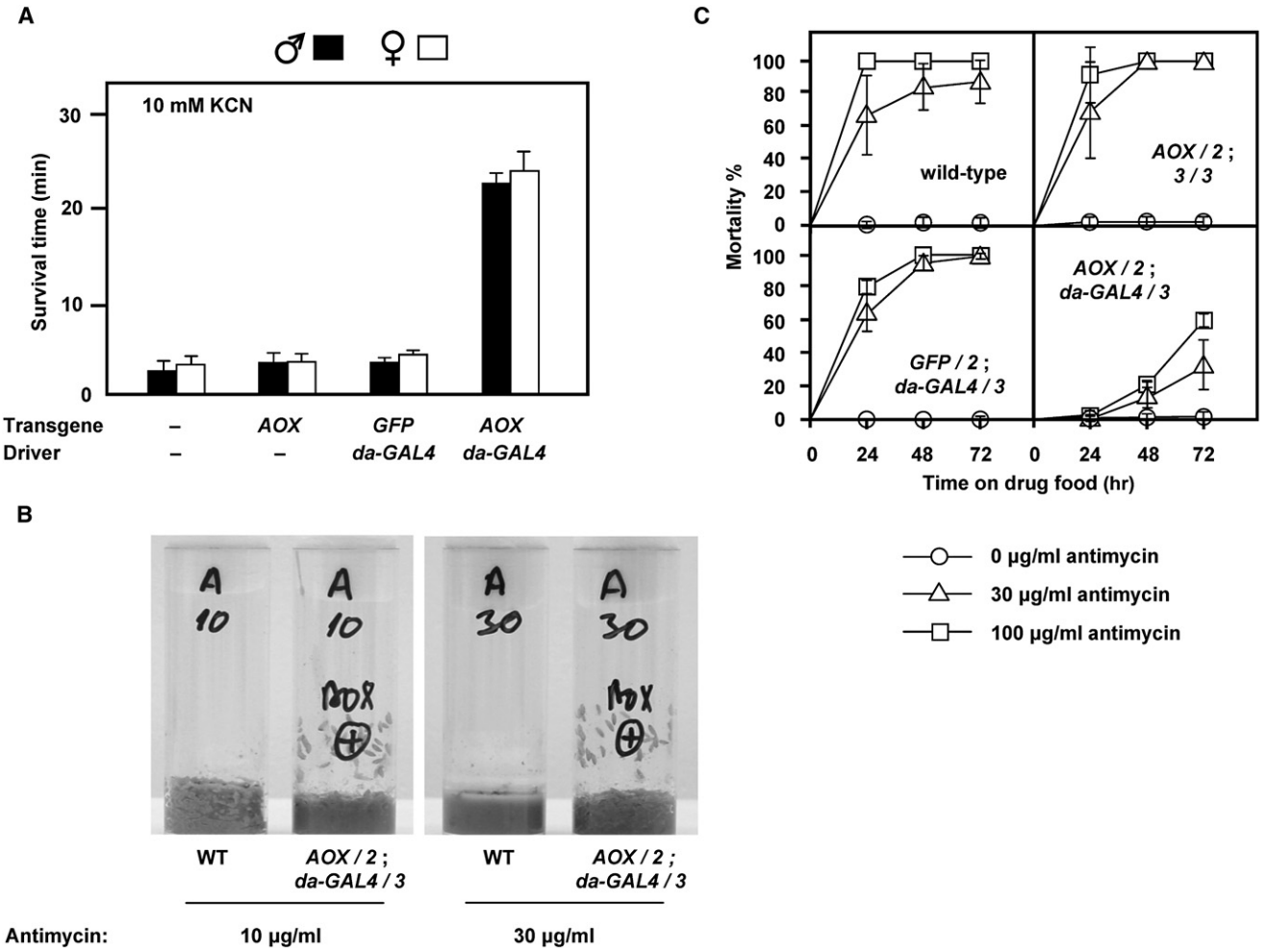

Figure 5. AOX-Expressing Flies Are Resistant to Cyanide and Antimycin

(A and B) Flies of genotypes indicated (nomenclature as in Figures 2 and 3; transgene and driver hemizygous where present), exposed to drugs as described in Experimental Procedures. Exposure was to 10 mM KCN. Mean survival time ± SD for batches of >100 of sex and genotype is indicated (A). Development on antimycin is shown in (B); food vials at 5 days postfertilization, containing antimycin at the concentrations indicated, and larvae of genotypes as shown. On antimycin-containing food, when AOX-expressing flies were at larval stage L3, wild-type flies failed to survive embryogenesis (at 30 µg/ml) or arrested in early larval stages (at 10 µg/ml). AOX-expressing progeny survived to eclosion, but with a 3–5 day developmental delay. For quantitation, see Figure S2J.

(C) Exposure of adult flies to antimycin-containing medium, as indicated. Data are from 12 replicate vials of each genotype and drug concentration shown, containing initially 15 males or virgin females. Data (means ± SD) from the two sexes were statistically indistinguishable, so are here pooled.

AOX Complements a Parkinson's Disease Model

Reasoning that AOX expression, by helping to maintain the respiratory chain in the oxidized state, may mitigate ROS production resulting from transient OXPHOS interruption, we investigated its effects in a *Drosophila* model of Parkinson's disease associated with defects in ROS handling, i.e., the mutant *dj-1*β. The human homolog (*DJ1*) has been identified as the disease gene in some cases of familial Parkinson's disease, and the fly mutant exhibits progressive locomotor decline (Park et al., 2005).

To test for complementation of *dj-1*β, we measured locomotor ability of wild-type and mutant flies over a period of 4 weeks posteclosion as well as of *dj-1*β flies hemizygous for AOX, plus or minus each of two GAL4 drivers, *nrv2-GAL4* (nervous-system-specific) and ubiquitous *Act5C-GAL4* (Figures 7A and S4A). We confirmed the previously reported locomotor defect of *dj-1*β and found that the presence of the AOX transgene, even without a driver, conferred a partial rescue. Rescue was substantially enhanced by ubiquitous or nervous-system-directed AOX expression. In AOX-expressing males, the *dj-1*β locomotor defect was almost completely abolished. The drivers alone conferred no such rescue.

To determine whether rescue of *dj-1*β was related to alleviation of oxidative stress, we measured ROS production in mitochondrial suspensions from flies aged 20–22 days, a time point at which AOX-nonexpressing *dj-1*β flies manifested a substantial locomotor defect. Mitochondria from flies of the various genotypes studied gave state 3 substrate oxidation and respiratory control index similar to those of aging AOX-expressing and wild-type flies studied earlier (Figure S4B). However, ROS production, measured as released hydrogen peroxide, was significantly higher in mitochondria from *dj-1*β mutant flies

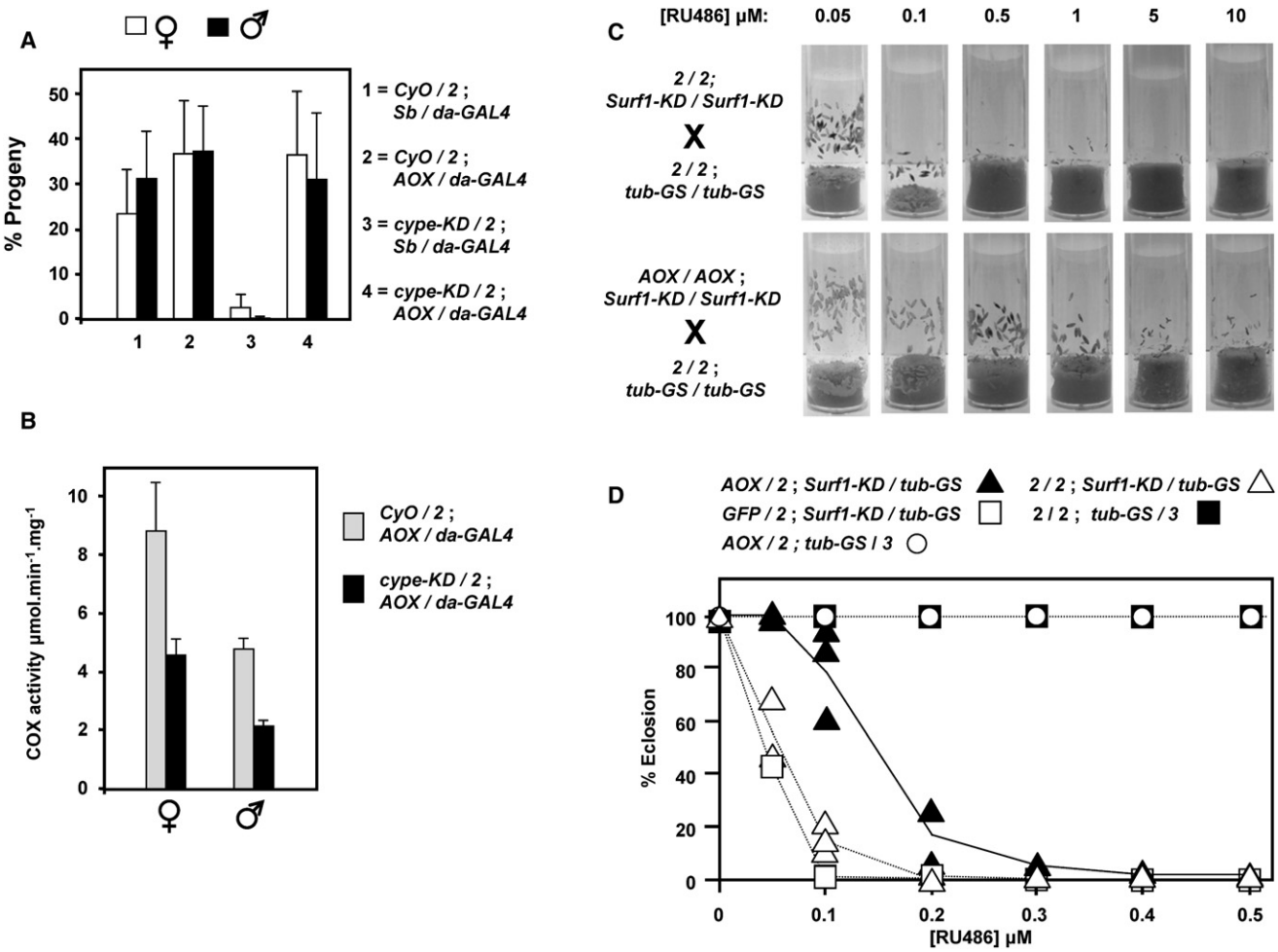

Figure 6. AOX Rescues Semilethality Due to COX Deficiency

(A) Proportion (mean percentages ± SD; three replicate experiments) of progeny of each genotype indicated, eclosing from cross between *da-GAL4* homozygous females and males of genotype *cype-KD / CyO ; AOX^{F24} / Sb*. Nomenclature is as in Figure 2; *cype-KD* encodes GAL4-dependent *cyclope*-directed dsRNA (Dietzl et al., 2007). *Sb* and *CyO* markers enable the progeny classes to be distinguished. Flies were cultured at 18°C with a 2 day heat shock at 30°C during larval stage L3 (see also Figures S3A and S3B). In parallel experiments, using *CG3731-KD* in place of *cype-KD*, flies equivalent to only the first two progeny classes eclosed (data not shown). CG3731 serves a dual function as core subunit 1 of OXPHOS complex III and the β subunit of the mitochondrial matrix processing peptidase, hence its knockdown has pleiotropic effects not rescued by AOX.

(B) COX-specific activity in AOX-expressing flies from the cross, either with or without concomitant *cype* knockdown. For same data normalized against citrate synthase activity, see Figure S3D. Means are ± SD.

(C and D) Crosses showing survival of flies hemizygous for *Surf1-KD* (encoding GAL4-dependent *Surf1*-directed dsRNA) and *tub-GS* transgenes, each on chromosome 3, with or without hemizygous *AOX^{F6}* or *GFP* as an additional GAL4-dependent transgene on chromosome 2, cultured at different doses of the inducing drug RU486. Image of actual vials is shown in (C), in which AOX-expressing flies consistently reach further in development than nontransgenic flies at any given drug dose. Percentage of pupae eclosing from parallel crosses, of the genotypes indicated, is also shown (D). Data from three different experiments superimposed.

than from wild-type flies of a given sex (Figure 7B). The presence of the unexpressed AOX transgene alone did not significantly mitigate this excess ROS production, but ubiquitous AOX expression decreased ROS production to wild-type levels (Figure 7B).

DISCUSSION

We previously demonstrated that the expression of *Ciona intestinalis* AOX can support cyanide-independent respiration and complement OXPHOS inhibition in cultured human cells (Hakkaart et al., 2006). The present work demonstrates that this applies also to a whole-organism model, *Drosophila*, in vivo.

AOX Expression Is Benign

Ubiquitous AOX expression in *Drosophila* at the level of a typical abundant mRNA appears to be benign. The protein is stable, is correctly targeted to mitochondria, and confers substantial and significant cyanide resistance to mitochondrial substrate oxidation in vitro. As in human cells, it appears to be enzymatically inert in the absence of an OXPHOS inhibitor (Figure S2H) and thus should not contribute significantly to electron flow under

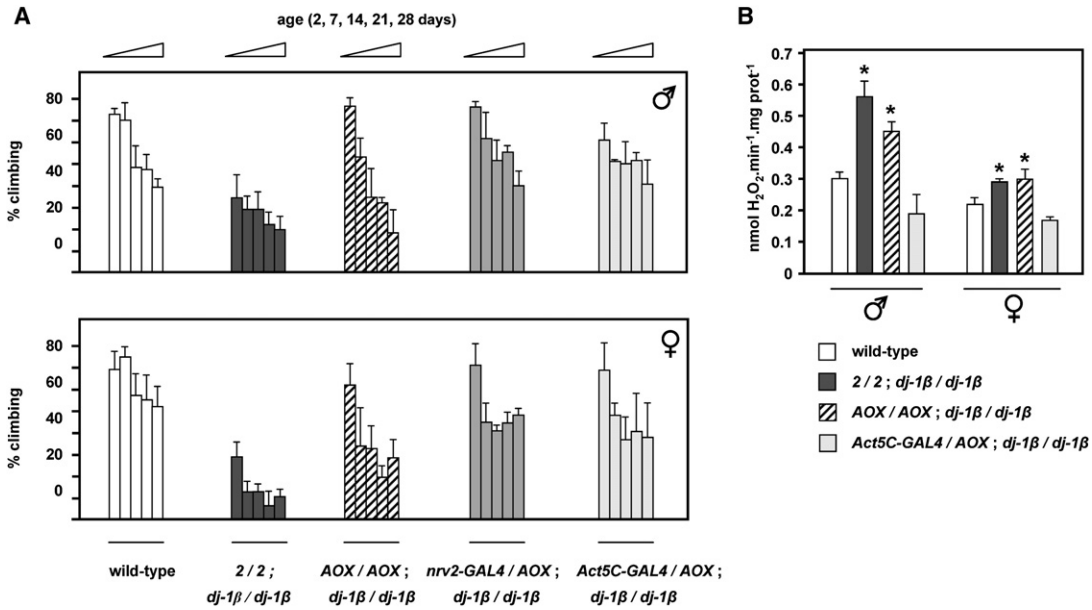

Figure 7. AOX Expression Rescues the *dj-1*β Mutant Phenotype

(A) Locomotor (climbing) assay, flies of sex and genotypes indicated, in the (w^{1118}) background; nomenclature as in Figure 2. The AOX transgene, where present, was derived from transgenic line F6 (insertion on chromosome 2). *dj-1*β is on chromosome 3, whereas the *nrv2-GAL4* and *Act5C-GAL4* drivers are carried on chromosome 2. Each set of bars (left to right) corresponds with a series of assays at 2, 7, 14, 21, and 28 days posteclosion. Means are ± SD from at least two and usually three or four replicate experiments, using 12–18 vials of ten flies of each sex and genotype.

(B) ROS (H_2O_2) production in mitochondria from flies of the sex and genotypes indicated. Plotted data are means ± SEM of at least five biological replicates. Asterisks denote data classes statistically different from other data classes, based on one-way ANOVA (Newman-Keuls test); males and females analyzed separately.

normal physiological conditions nor diminish ATP production by a substantial amount. Consistent with this, it had only a minimal effect on development (Figure 3A), with a slight exaggeration of the weight loss experienced by young adult flies (Figure 3B), suggestive of only a small drop in the overall efficiency of catabolism. A slight impairment thereof might nevertheless be sufficient to account for the evolutionary loss of AOX in both vertebrates and arthropods, organisms where maximal muscle-force generation, dependent on aerobic ATP supply, may be needed to escape predators or catch prey. Among metazoans, AOX genes have so far been found only in phyla composed of slow-moving or sessile organisms. AOX-expressing flies were also fertile. Although pUAST-derived transgenes are not expressed in the female germline (Rørth, 1998), AOX expression clearly does not compromise any somatic functions required for fertility.

AOX Rescues Lethality of Partial COX Deficiency

Resistance to antimycin and cyanide indicates that AOX can support at least a portion of the electron flow to oxygen when the cytochrome chain is inhibited in vivo. Cyanide is a wide-spectrum reversible inhibitor of oxygen-binding hemoproteins, including hemoglobin as well as COX. Although a globin homolog is present in insects (Hankeln et al., 2002), it is thought not to be involved in primary electron transport. The fact that AOX-expressing flies can develop on antimycin-containing medium indicates that proton pumping at complex I, plus some residual activity of the cytochrome chain, is sufficient to maintain ATP production at a rate that supports life. Importantly, although AOX is not itself proton pumping, it should nevertheless facilitate increased ATP production (at complex I or through substrate-level phosphorylation) under conditions of OXPHOS inhibition. It should also restore redox homeostasis and metabolic flux. AOX-expressing flies grown on antimycin-containing medium nevertheless showed a considerable developmental delay, a phenotype shared with mutants affecting OXPHOS, such as *technical knockout* (Toivonen et al., 2001), *stress-sensitive B* (Zhang et al., 1999), and *knockdown* (Fergestad et al., 2006). This delay may therefore be interpreted as a signature of bioenergy limitation during development.

The complete elimination of COX (e.g., via null alleles of *cyclope,* or effected by *Surf1* knockdown at high concentrations of RU486) is lethal even in the presence of AOX. This implies that proton pumping through complex I alone, maintained by the action of AOX handling downstream electron flow, nevertheless supplies insufficient ATP to maintain life. Note, however, that assembled complex IV may be required for efficient assembly or stabilization of the other OXPHOS complexes (Boekema and Braun, 2007), so the COX-deficient phenotype might in reality be pleiotropic. Partial knockdown of *cyclope*, which diminished COX activity by more than 50% in adult flies, was rescued by AOX, which gives an indication of the degree of COX defect that could potentially be compensated therapeutically. Similarly, AOX was able to rescue the pupal lethality of partial *Surf1* knockdown. The effect cannot be attributed to competition between the GAL4-dependent promoters, since the amount of knockdown was unaffected by coexpression of AOX (Figure S3F), and an inert GAL4-dependent transgene (GFP) produced no rescue (Figures 6D and S3G).

Surf1 is a conserved gene involved in complex IV assembly, but its null phenotype varies greatly between organisms. Suppressor analysis in yeast (Barrientos et al., 2002) implicated the Surf1 homolog in an early step of complex IV assembly. However, the interference with respiration is greater than can be accounted for by complex IV deficiency alone (Mashkevich et al., 1997), suggesting a more global role in organization of the respiratory membrane or coupling between complexes III and IV. Loss of *SURF1* function in humans causes COX-deficient Leigh Syndrome (Zhu et al., 1998; Tiranti et al., 1998; Yao and Shoubridge, 1999), but in the mouse, functional ablation of *Surf1*, while provoking a decreased COX activity (Dell'agnello et al., 2007), has virtually no physiological phenotype, supporting the notion that *Surf1* may have additional roles. In *Drosophila*, when knockdown was targeted specifically to the nervous system, impaired larval locomotion and adult optomotor responses were found, even though the decrease in COX activity in the brain was modest (Zordan et al., 2006). Although *SURF1*/*SHY1* mutations in humans and yeast are recessive, the amount of *Surf1* knockdown required to produce lethality in flies is only 65% at the RNA level (Zordan et al., 2006). In the present study, a knockdown of 40%–50% at the RNA level was sufficient to cause semilethality, although knockdown may be more efficient at the protein level (Zordan et al., 2006). It will be interesting to determine the exact degree of *Surf1* knockdown that AOX can compensate and the concomitant effects on OXPHOS biochemistry. To this end, transgenic flies constitutively expressing AOX without the need for a driver will be a useful tool.

AOX and Parkinson's Disease

AOX expression was able to complement also the phenotype of a *Drosophila* strain carrying a mutation in a homolog of a human Parkinson's disease gene (*DJ1*). Although the pathological mechanism of *DJ1* mutations is poorly understood, there is a general consensus that DJ1 deficiency entrains increased susceptibility to mitochondrial oxidative stress, particularly in the vulnerable dopaminergic neurons of the *substantia nigra* (Beal, 2007; Tan and Skipper, 2007). DJ1 has been postulated as both a sensor and a scavenger of ROS (Mitsumoto and Nakagawa, 2001; Taira et al., 2004; Canet-Aviles et al., 2004), and *Dj1* null mice are sensitive to oxidative stress (Kim et al., 2005), as are *Drosophila* mutants in either *dj-1*β or *DJ-1*α (Park et al., 2005; Menzies et al., 2005; Yang et al., 2005; Lavara-Culebras and Paricio, 2007). DJ1 has been suggested to function as a peroxiredoxin (Andres-Mateos et al., 2007) or as a chaperone (Zhou et al., 2006) for alpha-synuclein, whose aggregation it prevents (Batelli et al., 2008). It also protects against oxidative damage in cerebral ischemia (Aleyasin et al., 2007). AOX rescue of *dj-1*β is consistent with the idea that ROS overproduction caused by mitochondrial dysfunction is a common underlying feature of Parkinson's disease etiology (Beal, 2007; Tan and Skipper, 2007; Dimauro and Schon, 2008). Transient interruptions of electron flow may result in episodic bursts of excess ROS production. The AOX bypass would prevent such disturbances, decreasing net ROS production to levels that are no longer pathological, despite the failure of the detoxification system provided by *dj-1*β. A second possibility would be that AOX eliminates mitochondrial ROS directly, e.g., using semiquinone radicals as substrate. AOX is induced in *Euglena*, along with many antioxidant enzymes, by treatment with Cd^{2+} (Castro-Guerrero et al., 2008), a pro-oxidant (Watanabe et al., 2003), and its overexpression in plants protects from cold-induced oxidative stress (Sugie et al., 2006).

Low-level AOX expression in the absence of driver gave a transient locomotor rescue (Figures 7A and S4), even though its effects on mitochondrial ROS production were modest. AOX expression driven by *nrv2-GAL4*, which is highly restricted to the nervous system (Sun et al., 1999), completely rescued the locomotor defect. AOX expression directed only by the pUAST promoter in some specific but critical cells of the nervous system may thus be sufficient for rescue, even though, when ROS production is assayed in extracts prepared from the whole fly, the effect is diluted. Alternatively, since the beneficial effects of low-level AOX expression were seen only in young flies, effects on ROS production might be detectable only early in development.

Conclusion: The Therapeutic Potential of AOX

We showed previously that AOX can alleviate many deleterious consequences of OXPHOS dysfunction in cultured cells (Hakkaart et al., 2006). Here, we demonstrate that its expression is well tolerated in a whole organism lacking endogenous AOX, alleviating the effects of toxins or mutations affecting the cytochrome chain. Two such mutations represent *Drosophila* models of different classes of human OXPHOS disease, namely COX-deficient Leigh Syndrome (*Surf1* knockdown) and familial Parkinson's disease (*dj-1*β). The next step in testing whether AOX could have therapeutic utility would be to express it in mammalian models. The findings with *Drosophila* suggest that it could be of benefit in a wide spectrum of OXPHOS disorders. However, many technical obstacles will need to be overcome to make this a feasible therapeutic strategy, such as optimizing the enzyme for physiological temperature and oxygen levels and addressing its possible antigenicity.

EXPERIMENTAL PROCEDURES

Drosophila Stocks and Maintenance

Wild-type, w^{1118} mutant, standard balancer, driver, and RNAi lines were obtained from stock centers, with other lines derived or sourced as indicated in Table S1, which also gives details of all genotypes. Flies were maintained in standard medium with supplements (see Supplemental Data). For testing of resistance to antimycin, the drug (Sigma) was added to fly food at different concentrations. For testing of resistance to cyanide, 1 day old adult flies were placed inside plugged vials of 1% agarose containing 10 mM KCN, 50 mM Tris-HCl (pH 7.5) inside a fume hood at room temperature. After flies stopped moving, they were transferred to empty vials to check for recovery overnight. For induction of expression using the *tub-GS* driver, flies hemizygous for both the driver and the GAL4-dependent transgene(s) of interest were cultured in the presence of appropriate doses of RU486 (Mifepristone; Sigma), as indicated in figure legends.

Construction of AOX Transgenic *Drosophila* Lines

The *C. intestinalis* AOX cDNA, originally cloned in mammalian expression vector pCDNA5/FRT/TO (Hakkaart et al., 2006), was excised and recloned into a modified version of pGREEN-H-Pelican (Barolo et al., 2000) (*Drosophila* Genomics Resource Center; Bloomington, IN), placing it under the control of GAL4 supplied *in trans* (for full details, see Figure S1A). All restriction digestions were carried out under manufacturer's recommended conditions (New England Biolabs; Fermentas). The myc-tagged AOX cDNA was similarly recloned from the original mammalian expression vector (Hakkaart et al.,

2006). Both constructs were sequenced to verify the construction and the absence of mutations. Following microinjection into w^{1118} recipient embryos (VANEDIS *Drosophila* Injection Service; Oslo), transgenic progeny were established as independent lines in the w^{1118} background. Insertion sites were determined by inverse PCR (Toivonen et al., 2003) (see Supplemental Data for further details, including insertion sites). Selected lines retained were maintained as hemizygotes using the eye-color marker and appropriate balancers and used to generate homozygotes as needed.

Isolation of Mitochondria

Mitochondria were prepared essentially as described by Miwa et al. (2003), from batches of 150–200 flies ground in 0.5 ml isolation medium, with final resuspension in 50 μl isolation medium without BSA. Protein concentrations were determined using the Bradford assay (Bradford, 1976). For full details of subcellular fractionation used for western blotting, see Supplemental Data.

Polarography

Mitochondrial substrate oxidation rates were measured by polarography using a Clark type oxygen electrode (Hansatech Instruments; Norfolk, UK) in a final volume of 0.5 ml at 25°C. Mitochondria (between 0.25 and 0.5 mg/ml) were incubated in incubation buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% BSA [pH 7.2]) supplemented with either 20 mM sn-glycerol-3-phosphate (plus 5 μM rotenone; Sigma) or a mixture of 5 mM sodium pyruvate and 5 mM proline as substrate, followed by the addition of 1 mM ADP, with subsequent addition of 100 μM KCN or 10 μM SHAM (Sigma) to test the effect of AOX on substrate oxidation.

Enzymatic Analysis

The activities of OXPHOS complexes I + III and II + III were measured as described previously (Fernández-Ayala et al., 2005). Citrate synthase activity was determined by the reduction of dithio-bis-nitrobenzoic acid (DTNB) followed at 412 nm (extinction coefficient = 21,000 M^{-1}/cm^{-1}) in a reaction containing 100 mM Tris-HCl, 2.5 mM EDTA, 37 μM acetyl-CoA, 75 μM DTNB, and 300 μM oxaloacetate (pH 8.0). COX activity was measured in 40 mM sodium phosphate buffer (pH 7.5) by following the disappearance of thiosulfite-reduced cytochrome *c* (initially 25 μM) at 550 nm (extinction coefficient = 27,800 M^{-1}/cm^{-1}). Specific activity was determined by subtracting the rate in the presence of 2 mM KCN from the rate without the inhibitor.

Measurement of Mitochondrial ROS Production

Mitochondrial free radical production was determined by measuring the generation of hydrogen peroxide in solution in the presence of superoxide dismutase, as described previously (Sanz and Barja, 2006), and adapted to flies (Miwa et al., 2003). For further details, see Supplemental Data. Known amounts of H_2O_2 generated in parallel by glucose oxidase with glucose as substrate were used as standards. All experiments were repeated in the absence of substrate, and background fluorescence changes were subtracted.

RNA Extraction and Quantitation

For expression analysis, homozygous AOX transgenic line F6 females were crossed either to males homozygous for the da-GAL4 or *tub-GS* driver or to males hemizygous for the Act5C-GAL4 driver balanced against *CyO*. Total RNA was extracted using the Trizol method from 100 mg of adult flies or L3 larvae anesthetized on ice and then frozen at −80°C, followed by treatment with RNase-free DNaseI (for full details, see Supplemental Data). Quantitative RT-PCR was carried out under standard conditions using hybridization probes specific for AOX, RpL32, and GAPDH (Tib-MolBiol; Berlin) on a Roche Diagnostics LightCycler 1.5 (for full details, see Supplemental Data).

In Situ Hybridization

Whole-mount in situ hybridization was performed essentially as described by Fernández-Moreno et al. (2007), with minor modifications as indicated in the Supplemental Data.

Protein Extraction and Western Blotting

Protein extraction and western blotting used standard reagents and procedures as detailed in Supplemental Data. Custom-manufactured anti-AOX antibody (rabbit polyclonal) was supplied by 21st Century Biochemicals (Marlboro, MA).

Behavioral Analysis

Locomotor activity was determined in two ways. Climbing ability was assayed using a modified version of the procedure of Cha et al. (2005), as detailed fully in Supplemental Data. The second method used a combined bang-sensitivity/climbing assay. Aliquots of 20 adult female flies aged 20–22 days were vortexed at the maximum setting for 30 s, after which the percentage of flies climbing 7 cm in 15 s was recorded.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, and four figures and can be found online at http://www.cell.com/cell-metabolism/supplemental/S1550-4131(09)00064-3.

ACKNOWLEDGMENTS

This research was funded by Academy of Finland, Tampere University Hospital Medical Research Fund, Sigrid Juselius Foundation, CNRS, INSERM, Leducq Foundation, AMMi, Telethon multicenter grant GGP07019, the Government of Andalucia (Postdoctoral Fellowship to D.J.M.F.-A.), and EMBO (Long-Term Fellowship to A.S.). We thank Outi Kurronen, Tuomas Brock, and Rami Kurimo for technical assistance; Placido Navas for facilities and resources; Mikko Soikkeli and Shweta Manjiry for advice on in situ hybridization; Esko Kemppainen for *Drosophila* maintenance and advice on cloning, insertion-site mapping, and crosses; Janne Toivonen and Scott Pletcher for the tub-GS driver; Vienna Drosophila RNAi Centre for the *cyclope* knockdown line; and Eric Dufour, Emmanuel Dassa, and Anu Suomalainen for many useful discussions.

Received: July 22, 2008
Revised: November 27, 2008
Accepted: March 11, 2009
Published: May 5, 2009

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