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Ciona intestinalis NADH dehydrogenase NDX confers stress-resistance and extended lifespan on *Drosophila*

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ABSTRACT

An assembled cDNA coding for the putative single-subunit NADH dehydrogenase (NDX) of *Ciona intestinalis* was introduced into *Drosophila melanogaster*. The encoded protein was found to localize to mitochondria and to confer rotenone-insensitive substrate oxidation in organello. Transgenic flies exhibited increased resistance to menadione, starvation and temperature stress, and manifested a sex and diet-dependent increase in mean lifespan of 20–50%. However, NDX was able only weakly to complement the phenotypes produced by the knockdown of complex I subunits.

1. Introduction

Many mitochondrial diseases are associated with defects in complex I (cI) of the electron-transport chain. In mammals (e.g. bovine), this complex consists of 45 subunits encoded by separate genes, seven of which are in the mitochondrial genome [1–3]. Mutations in these genes lead to diverse pathologies affecting the central nervous system, sensory organs, and skeletal and heart muscle, which may manifest at any stage of life. Disease may also result from mutations in genes encoding proteins responsible for the assembly of this multi-subunit complex. Ageing has also been linked to the functional decline of cI [4,5].

At this time, cI-linked disease remains incurable, with only palliative treatments available. As with other diseases of genetic origin, the development of somatic gene therapy would be highly desirable, but faces severe practical obstacles, in addition to the safety and efficacy issues that affect all such strategies. First, its genetic heterogeneity is currently a challenge even for diagnosis. Whilst establishing the broad category of cI deficiency is relatively straightforward [2], the nature of the underlying gene defect can remain unclear, even if the whole coding genome is sequenced. This reflects the fact that many components of the machinery of cI biosynthesis are still unidentified. Second, no effective technology for editing of mtDNA has yet been established; hence the many cases linked to mtDNA-encoded subunits of cI would require a different approach. The hydrophobic nature of the mtDNA-encoded subunits also presents challenges for allotopic expression. Third, the diversity and multiplicity of affected tissues, including the brain, require a broad-spectrum approach that in some cases would need to be effective right from birth, if not before. Fourth, many mitochondrial diseases manifest defects in multiple complexes of the respiratory chain, due to genetic lesions affecting the machinery of mtDNA maintenance or expression. These cases are as common as those affecting only cI. Genetic therapy thereof requires either direct repair of the underlying lesion, which is again highly heterogeneous, or else an approach targeting multiple components of the respiratory chain.

An attractive strategy to overcome many of these problems is suggested by the fact that fungi, plants, and some animals contain the components of an alternative mitochondrial respiratory chain, where the function of cI is replaced by one or more single-subunit, non-protonmotive NADH dehydrogenases encoded in nuclear DNA. These enzymes are embedded in the inner mitochondrial membrane, but adopt two different topologies. The so-called 'external' NADH dehydrogenases, such as Nde1 and Nde2 in yeast (*Saccharomyces cerevisiae*), or the sole representative of the superfamily in *Yarrowia lipolytica*, YLNDH2, oxidize NADH supplied directly from the cytosol, whereas internal NADH dehydrogenases such as *S. cerevisiae* Ndi1 face the mitochondrial matrix, and use substrate supplied from there [6]. These alternative dehydrogenases also differ according to whether they use NADH or NADPH (or both) as their substrate [6,7].

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Expression of yeast Ndi1 has been successfully used for alleviation of cI defects in multiple models including nematodes [8], fruit flies [9,10], rats [11,12], and human cells [13]. Additionally, yeast Ndi1 has been shown to have therapeutic benefit in several Parkinson's disease models [14–19] and evidence supporting a possible use in anti-cancer treatment has also been put forward [20]. Many other physiological effects were also reported in model organisms expressing yeast Ndi1, including extended lifespan [9,21] and resistance to particular stresses [9]. Intriguingly, the alternative NADH dehydrogenases show some sequence similarity with AIF (apoptosis-inducing factor) and the *S. cerevisiae* Ndi1 protein has been shown to be capable of inducing apoptosis [22,23].

Recently, the components of the alternative respiratory chain, including a rotenone-insensitive NADH dehydrogenase, have been found in several animal taxa, including tunicates (urochordates) [24]. However, the properties of these alternative NADH dehydrogenases remain uninvestigated, despite an accumulating literature on the alternative respiratory chain of some animals and protozoans, based on our own studies and those of others [25–31]. Our laboratory has, for example, exploited the alternative oxidase (AOX) from the tunicate *Ciona intestinalis* to by-pass defects in complexes III and IV, by expressing it in fruit fly [25,26], mouse [27] and human cell [28–30] models.

Whilst the therapeutic potential of alternative NADH dehydrogenase has been demonstrated on diverse models, using *S. cerevisiae* Ndi1 [8–21], the yeast enzyme may not be optimal for such use, for several reasons. First, and most importantly, it exists in vivo without the presence of cI. Strictly therefore, in its host organism, it is not an alternative to cI, but its replacement. Conversely, it co-exists with external NADH dehydrogenases Nde1 and Nde2, which may share some of its metabolic roles. Any properties of alternative NADH dehydrogenases that relate to these interactions and, most importantly, co-regulation with cI, are likely to be altered or missing in the yeast enzyme. Second, in accordance with this prediction, yeast Ndi1 appears to be constitutively active [9], unlike AOX from *C. intestinalis*, which only contributes to electron flow conditions where the regular respiratory chain is inhibited [28]. Third, most likely as a consequence of constitutive activity, yeast Ndi1 expression can also be deleterious, under conditions where the ATP supply is limiting. This was found to be the case in two *Drosophila* models of mitochondrial disease, created by the knockdown of complex IV subunits [26] or the tko^{25t} mutation affecting mitochondrial protein synthesis [32]. Finally, whereas animals such as *C. intestinalis* have a single NADH dehydrogenase, yeast has three and plants typically several more. Ndi1 must therefore co-operate or compete metabolically with other enzymes, and may thus be adapted to handle some physiological stresses but not others.

In order to overcome these limitations, and implement a cI by-pass from a source phylogenetically closer to humans, we set out to characterize the alternative NADH dehydrogenase from *C. intestinalis* (here designated NDX) by expressing it in *Drosophila* and analyzing the properties it confers. Specifically, we tested its intracellular targeting, its ability to replace cI both in organello and in vivo, and the phenotypic properties it confers upon flies.

2. Materials and methods

2.1. Cloning procedures

C. intestinalis expressed sequence tag (EST) cDNA clones cibd016*c*12, ciad062*k*07 and ciem809*l*11 were obtained from RIKEN BioResource Center (Tsukuba, Japan). The full NDX coding-region was assembled by serial overlap-extension PCR (Supplementary Fig. S1A, B), using Phusion F-530S high-fidelity DNA polymerase (Thermo Scientific), with addition of terminal restriction sites *Sal*I and *Nhe*I to facilitate cloning. S.N.A.P.™ UV-Free Gel Purification Kit (Life Technologies) was used for DNA recovery. Following initial cloning into ZeroBlunt TOPO vector (Life Technologies) NDX was recloned into vector pMT/V5-His B (Life Technologies) for expression in S2 cells, following amplification with chimeric primers pMT_Nhe1cK_F and pMT_Sal1cK_R (Table S1) to introduce appropriate restriction sites (Fig. S1C). Recloning into vector pUASTattB (Addgene, [33]), to create the transgenic construct pUASTattB-NDX, used the scheme shown in Fig. S1D. Positive clones were selected by colony PCR, then verified by restriction analysis and sequencing. Empty vector pUASTattB or pUASTattB-NDX was injected into Bloomington Drosophila Stock Center lines 24861 (y^1 *M{vas-int.Dm}ZH-2A* w^*; *PBac{*y^+-attP-3B}VK00001) and 24871 (y^1 *M{vas-int.Dm}ZH-2A* w^*; *PBac{*y^+-attP-3B}VK00033) by Rainbow Transgenic Flies, Inc. (Camarillo, CA, USA), after which the construct *M{vas-int.Dm}ZH-2A* bearing φC31 recombinase was crossed out.

2.2. Drosophila stocks and maintenance

Except where stated, flies were maintained and grown on standard medium at 25 °C, using a 12 h light/dark cycle, as described previously [9,25]. Following characterization, transgenic lines were maintained over appropriate balancers for chromosome 2 (*CyO*) or 3 (*TM3Sb*). Ubiquitous expression was driven using a derivative of Bloomington line 8641 but with genotype y^1 w^*; *P{da-GAL4.w*[-]}3. RNAi targeted against cI subunits CG6020 and CG3683 used VDRC lines 13131 and 46797 (Vienna *Drosophila* RNAi Center), respectively, as described previously [4]. Crosses using flies transgenic for UAS-dependent *C. intestinalis* AOX employed line $UAS\text{-}AOX^{F24}$ (chromosome 3 insertion, [25]) over balancer *TM3Sb*.

2.3. Cell culture and expression assays

Drosophila S2 cells were grown in Schneider's medium at 27 °C. Co-transformation of vectors pMT/V5-His B (with integrated NDX) and pCoHygro, selection of a stably expressing cell-line and induction of NDX expression were performed using *Drosophila* expression system (DES®-inducible kit, Life Technologies) according to the manufacturer's instructions. After induction, cells were fixed and stained as described [34], using mouse anti-V5 (Life Technologies) and rabbit anti-COXIV (Abcam) as primary antibodies, respectively with AlexaFluor® 568 goat anti-mouse IgG (H+L) and goat anti-rabbit AlexaFluor® 488 IgG (H+L) (Life Technologies) as secondary antibodies. Protein extracts (from adult flies or S2 cells) were prepared, concentrations measured and Western blots carried out as described previously [25], using either the above anti-V5 antibody, with HRP-conjugated horse anti-mouse IgG (H+L) (Vector Laboratories) as secondary antibody, both at 1:10,000 dilution, or a customized rabbit antibody raised and purified against NDX peptide LPATAQVAERKGKWLAEY (21st Century Biochemicals) at 1:15,000 dilution, with reprobing for NDUFS3 (cI subunit) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase), all as described previously [9]. QRTPCR was carried out as described previously [25], using NDX-specific primers (Table S1) and RpL32 as a normalization control [25].

2.4. Respirometry

Mitochondria were isolated from 70 to 90 flies. Flies were immobilized on ice and gently homogenized at 4 °C in a chilled mortar in 1 ml of isolation buffer (250 mM sucrose, 5 mM Tris/HCl, 2 mM EGTA, 0.1% bovine serum albumin, pH 7.2). The homogenate was filtered through muslin and centrifuged for 5 min at 200 g_{max}. The supernatant was then centrifuged at 9000 g_{max} for 10 min to collect mitochondria. The pellet was resuspended in 50 μl of the isolation buffer without bovine serum albumin. Oxygen consumption was measured with a Clarke-type oxygen electrode (Oxygraph-2k, Oroboros) in a buffer containing 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, and 0.2% bovine serum albumin, pH 7.2, with successive additions of substrate (5 mM sodium pyruvate, 5 mM L-proline), 5 mM ADP, 0.5 μM rotenone and 1 mM KCN.

2.5. Sequencing of C. intestinalis genomic DNA

C. intestinalis specimens were obtained from the Station Biologique de Roscoff, France (Service Expédition de Modèles Biologiques, Centre de Ressources Biologiques Marines, CNRS). Dissected muscle of single individuals was stored at −80 °C prior to processing for DNA extraction by salting-out [35]. Segments of the NDX coding sequence were amplified and directly sequenced using various primers as detailed in Table S1.

2.6. Stress-resistance assays

Resistance to the reactive oxygen species (ROS) generator menadione was tested for batches of ten flies transferred into vials containing folded strips (2.4 × 12 cm) of four-layer cellulose filter paper soaked with 0.8 ml of 20 mM menadione sodium bisulfite (Sigma-Aldrich) in 5% sucrose. Survivors were counted every 12 h following the start of exposure [36,37]. Starvation resistance was measured similarly, except that the filter paper was soaked with water. Survival on agar-only diet was scored in vials containing solidified agar in place of standard food. To evaluate resistance to heat stress, single flies were transferred into small glass vials with cotton stoppers and placed in a water bath at 41 °C [36,38]. The time taken to reach coma was recorded. After heat shock, vials of paralyzed flies were placed, without further disturbance, at room temperature (23–25 °C), to score the time taken for full recovery of locomotion. About 20 males and 8–9 females were tested for each genotype, sex and age. Recovery from cold-induced paralysis, induced by placing flies at 4 °C for 15 min, was measured by the same procedure.

2.7. Lifespan curves

Lifespan was measured in mortality cages [36], constructed from 15 cm diameter piping. A plastic food vial was screwed to the cage through a hole in the side-wall. The food contained different concentrations of yeast and sucrose (see figure legends), plus 1.5% agar and 0.4% propionic acid. The number of dead flies was recorded every second day. Dead individuals were aspirated through a rubber-covered hole on the sidewall opposite the food-vial hole.

3. Results

3.1. Identification, assembly and cloning of NDX

We identified a *C. intestinalis* homologue of the fungal and plant non-proton-motive NADH dehydrogenases by BLAST analysis of publicly available cDNA (EST) and genomic sequence resources. Based on this analysis, only one such close homologue of the superfamily appears to be present in the *Ciona* genome, currently shown provisionally as NCBI database entry XP_002122465. No such homologue was found in arthropods. Using overlapping cDNA clones from a *Ciona* EST library (see Fig. S1), we were able to assemble a full-length cDNA by high-fidelity overlap-extension PCR. The full-length sequence (Fig. S2) predicts a polypeptide similar to yeast (*S. cerevisiae*) Nde1, Nde2 and Ndi1 or to *Arabidopsis* NDA1, NDA2, and NDB1–4. It is slightly more similar to Nde1/2 (38% identity) than to Ndi1 (34% identity), but also more similar to the NDA than to the NDB family in *Arabidopsis*. For this reason we provisionally designate it as NDX since, without detailed functional and topological information, it is impossible to state whether it acts on NADH or on NADPH or both, and whether it faces the mitochondrial matrix (like Ndi1 and NDA) or the intermembrane space (like Nde1/2 and NDB) or perhaps even serves both compartments.

Like most genes in *C. intestinalis* [39], NDX appears to be highly polymorphic, although most of the variation is at silent sites. The predicted amino acid sequence of the fully assembled cDNA differed from the database entry at two amino acids (Fig. S2B). In order to be sure that these variants were not due to a cloning or PCR artifact, we analyzed genomic DNA amplified from individual specimens of *C. intestinalis*. In both cases, the prevalent or sole allele was identical to our cloned cDNA, not to the database entry (Fig. S2B). Homozygosity at these positions indicates that these are either natural polymorphisms in the gene or errors in the genomic DNA sequence underlying the database entry. The expressed cDNA should thus be functional.

3.2. NDX is expressible in Drosophila and targeted to mitochondria

The NDX cDNA was recloned into the copper-inducible expression vector pMT with an in-frame epitope tag (V5) and transfected into *Drosophila* S2 cells. After induction, signal from the V5 tag was colocalized with COXIV by immunocytochemistry (Fig. 1A) and, based on Western blotting, co-fractionated with mitochondrial marker NDUFS3 (cI) but not cytosolic marker GAPDH (Fig. 1B). The extrapolated molecular weight of the polypeptide (49 kDa) is less than predicted for the full-length fusion protein including the V5 tag (55.9 kDa), and suggests that the 50 amino-acid targeting peptide predicted by Mitoprot is cleaved upon mitochondrial import.

To investigate its effects at the whole-organism level, NDX was introduced into the fly genome using the φC31 recombination system [33,40], under the control of the GAL4-dependent UAS promoter. Transgenic lines were created containing single insertions of *UAS-NDX* on each of chromosomes 2 and 3 (designated *UAS-NDX*^{2} and *UAS-NDX*^{3}, respectively) with parallel control lines containing insertions of the empty vector at the same positions to serve as controls (here designated con^2 and con^3). Upon global induction using the *da-GAL4* driver, each NDX-transgenic line was found to be viable when either hemizygous or homozygous for the transgene, with no apparent deleterious phenotype. GAL4-dependent transcription of NDX was verified by QRTPCR (Fig. 1C), showing expression in hemizygous flies approximately 50-fold greater than in uninduced homozygous flies, and a further 2–3 fold increased by a second copy of the transgene. Expression at the protein level was verified using a customized antibody raised against NDX peptides (Fig. 1D). Since NDX co-exists naturally with AOX, which provides an equivalent by-pass of respiratory chain complexes III and IV, we tested the possibility that the two enzymes when co-expressed might generate a lethal 'short-circuit' of oxidative phosphorylation. When NDX-expressing flies of either sex were crossed with *UAS-NDX* plus *UAS-AOX* double transgenic flies, viable progeny expressing both transgenes were produced, albeit in decreased numbers compared with controls (Fig. 2A). Flies expressing a single copy of each transgene showed an eclosion delay of slightly more than 1 day, which was increased by a further day if two expressed copies of NDX were present (Fig. 2B).

3.3. NDX confers rotenone-resistance to mitochondrial substrate oxidation

The functionality of expressed NDX was verified by polarography of mitochondrial suspensions supplied with a standard cI-linked substrate mix, in the presence or absence of rotenone. Mitochondria from NDX-expressing flies of both sexes showed a significant rotenone-resistant substrate oxidation compared with controls (Fig. 3A, B), which was completely abolished by the further addition of KCN (Fig. 3B). The activity varied in amount according to the number of copies of the *UAS-NDX* transgene and of the *da-GAL4* driver: two copies of each gave approximately 20% rotenone resistance in both sexes. Attempts to measure NADH dehydrogenase activity in vitro gave less consistent results. In sonicated mitochondrial extracts from NDX-expressing flies we observed a substantial rotenone-resistant activity, but this was not stable with time. No such activity was detectable using intact mitochondria.

3.4. NDX confers stress-resistance to Drosophila

The alternative respiratory chain is believed to confer resistance against stresses resulting in mitochondrial inhibition. We therefore

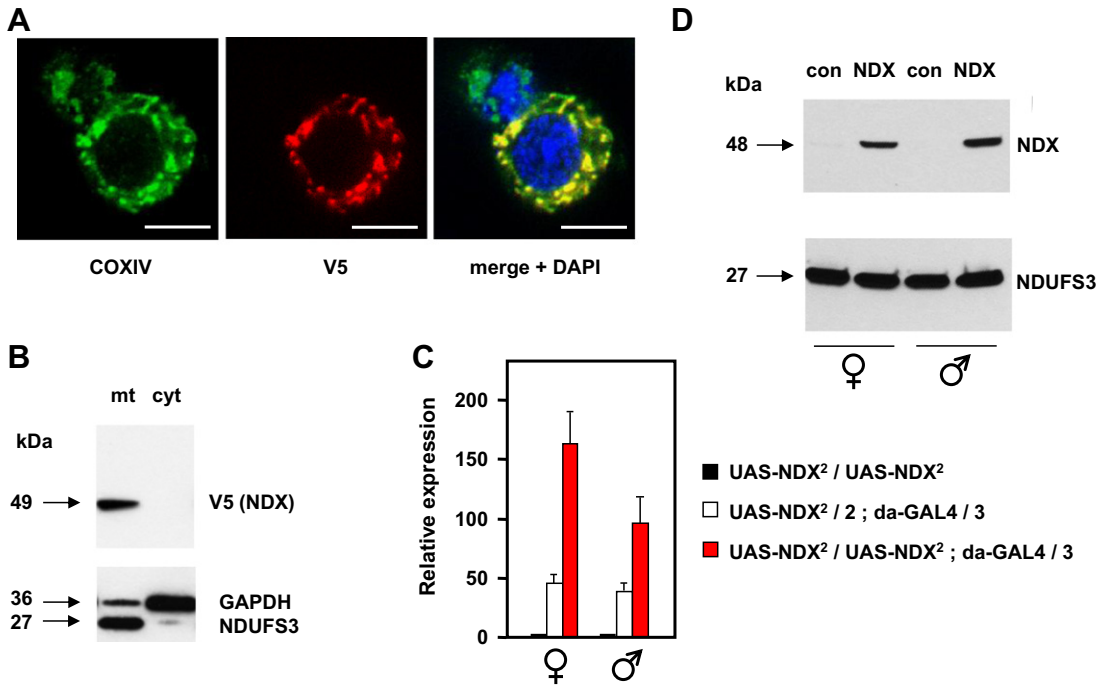

Fig. 1. Expression of NDX in *Drosophila*. (A) Immunocytochemistry of S2 cells induced to express transfected NDX-V5, probed with the antibodies indicated. Scale bar = 4 μm. Note the cell (top-left) staining only for COXIV, which represents an untransfected cell in the population. (B) Western blot of mitochondrial (mt) and cytosolic (cyt) fractions (150 μg) from S2 cells induced to express transfected NDX-V5, probed for V5 epitope and reprobed for GAPDH and NDUFS3 as markers for the two compartments. Molecular weight in kilodaltons as indicated, based on migration of markers. (c) Expression level of NDX in transgenic flies as indicated, based on QRTPCR normalized to ribosomal protein RpL32 (widely used for standardization, due to its ubiquitous expression), and then to the level in non-induced homozygous flies for NDX on chromosome 2 (denoted UAS-NDX^2). Genotypes showing 2 or 3 denote the corresponding wild-type chromosomes. Means ± SD from three independent biological replicates, all significantly different from each other (Student's t test, $P < 0.05$) for flies of a given sex. (D) Western blot of total protein extracts from NDX transgenic flies of the indicated sexes, induced to express NDX, versus control flies (con). Blots were probed for NDX then reprobed for NDUFS3 as loading control. An antibody raised and purified against a second NDX peptide, VQDFHLSHAVQLDPKSKTL, gave identical results.

tested the susceptibility of NDX-expressing flies to various such treatments. We first tested exposure to the ROS generator menadione, whose acute toxicity is believed to be due to overproduction of mitochondrial superoxide [41]. Freshly eclosed NDX-expressing females survived more than twice as long ($t_{1/2}$ = 91 h) as controls ($t_{1/2}$ = 40 h) in vials containing tissue paper soaked in 5% sucrose solution plus

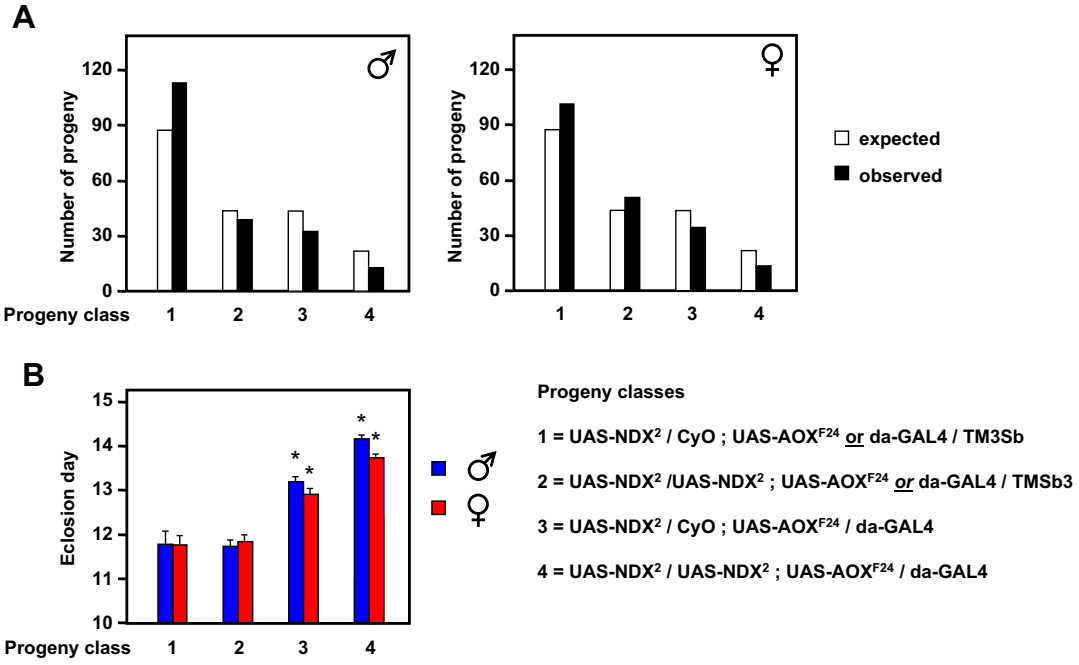

Fig. 2. Viability of NDX and AOX co-expressing flies. (A) Number of progeny of the indicated classes, from the cross *UAS-NDX^2 / CyO ; UAS-AOX^{F24} / TM3Sb* × *UAS-NDX^2 / CyO ; da-GAL4 / TM3Sb*. Pooled data from independent reciprocal crosses, that gave almost identical results. Observed frequencies of progeny classes were significantly different from expectation (χ^2 test, $P < 0.01$ for males, $P < 0.05$ for females). See Supplementary Data for full explanation of genetic notation and expected output. (B) Eclosion times for flies of the indicated progeny classes, means ± SD for 3 independent crosses. Asterisks indicate significant differences of other classes, from progeny class 1 of the given sex (Bonferroni-corrected t test, $P < 0.01$).

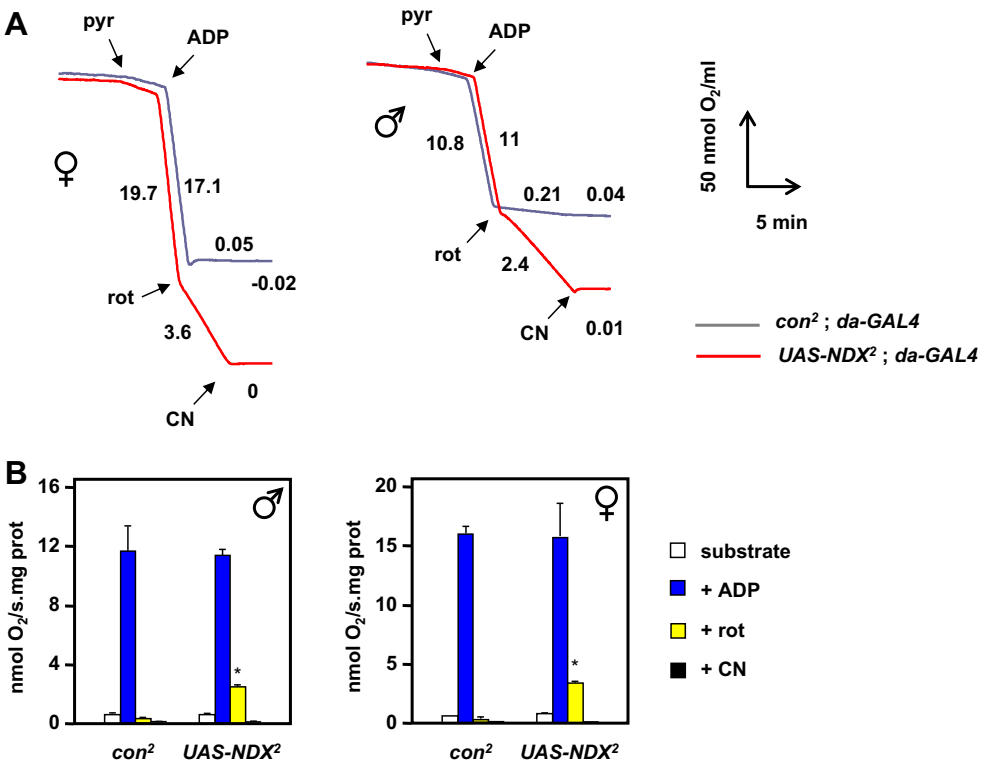

Fig. 3. Substrate oxidation by mitochondria from NDX-expressing flies. (A) Typical oxygraph traces for mitochondrial suspensions from flies of the indicated sexes and genotypes (*con²* denotes insertion of pUASTattB vector only, at the same position on chromosome 2). Arrows indicate addition of pyruvate + proline (pyr), ADP, rotenone (rot) and potassium cyanide (CN). (B) Rates of oxygen consumption by mitochondrial suspensions from flies of the indicated genotypes, also bearing two copies of *da-GAL4*, treated successively, as indicated. Asterisks indicate significant differences from mitochondria of control flies of the given sex ($P < 0.05$ by Student's t test).

20 mM menadione (Fig. 4A). Taking 36 h of exposure as a discriminating condition, we verified significant protection by NDX of flies of both sexes and different ages (Fig. 4B).

NDX-expressing flies were also significantly protected against thermal stresses. Both young and ageing NDX-expressing flies of both sexes showed significantly decreased recovery times from both cold- (4 °C) and heat- (41 °C) induced paralysis, as well as increased time of resistance to heat-shock before the onset of paralysis (Fig. 4C–E). NDX also conferred a modest resistance against total starvation (Fig. 4F), although the effect was not seen when flies were cultured on a minimal diet of just agar (Fig. S3A), on which they survived only for about 4–5 days.

3.5. NDX confers extended lifespan to Drosophila

In previous studies, the ubiquitous expression of yeast Ndi1 in *Drosophila* was shown to extend lifespan independently of dietary restriction [9]. Therefore, we tested whether NDX would have a similar effect. Compared with controls, NDX expression resulted in a significant increase (>50%) of both mean and maximum lifespan in rich media (Fig. 5A), although this effect was mitigated by dietary restriction (Fig. 5B, C). On a medium poor in both sugars and protein, the lifespan increase was only of the order of a few percent (Fig. 5C). Lifespan of males and females was increased to a similar extent (Fig. S3B, compare with Fig. 5B).

3.6. NDX weakly complements lethality due to complex I knockdown

Yeast Ndi1 expression was shown previously to complement the lethality (at 25 °C) of knockdown of subunits of cI [9] in *Drosophila*, whilst leaving a residual phenotype [32]. We therefore tested whether NDX was able to act similarly. At 25 °C, knockdown of *CG6020* or *CG3683*, *Drosophila* homologues of human *NDUFA9* and *NDUFA8*, respectively, driven by *da-GAL4*, was developmentally lethal, but co-expression of NDX was unable to rescue this lethality. At room temperature (21 °C) *CG3683* knockdown was still lethal, with a tiny number of escapers, but co-expression of two copies of NDX was sufficient to generate a substantial number of progeny (Table 1). At 21 °C *CG6020* knockdown was no longer lethal, but a similar co-expression of NDX enabled significantly more progeny knocked down for *CG6020* to eclose (Table 1), compared with controls not expressing NDX. Whilst these findings indicate a weak rescue, caution is still needed interpreting these findings, as discussed below.

4. Discussion

Using *Drosophila* as a model system, we provide here the first evidence for the functionality of a member of the single-subunit, non-proton-motive NADH dehydrogenases from a metazoan source.

4.1. Enzymatic activity of NDX

When expressed in *Drosophila*, *Ciona* NDX has properties indicating that it is a *bona fide* NADH dehydrogenase. The protein was targeted to mitochondria based on immunocytochemistry and western blots, and supported the rotenone-insensitive oxidation of cI-linked substrates added to mitochondrial suspensions. This activity was completely inhibited by cyanide, indicating that electron flow through NDX is functionally coupled to the downstream portion of the mitochondrial respiratory chain. The absence of detectable NADH dehydrogenase activity in intact mitochondrial suspensions is consistent with NDX being of the 'internal' type. However, the activity detected in sonicated extracts was unstable, hence unquantifiable. One possible explanation may be that the enzyme associates with cI and/or other respiratory chain complexes, which may regulate its activity. A final conclusion regarding the preferred substrate(s), topology and other properties of NDX must await the determination of a reliable procedure for its extraction in a functionally stable form. Until then, we prefer to retain the nomenclature NDX, which

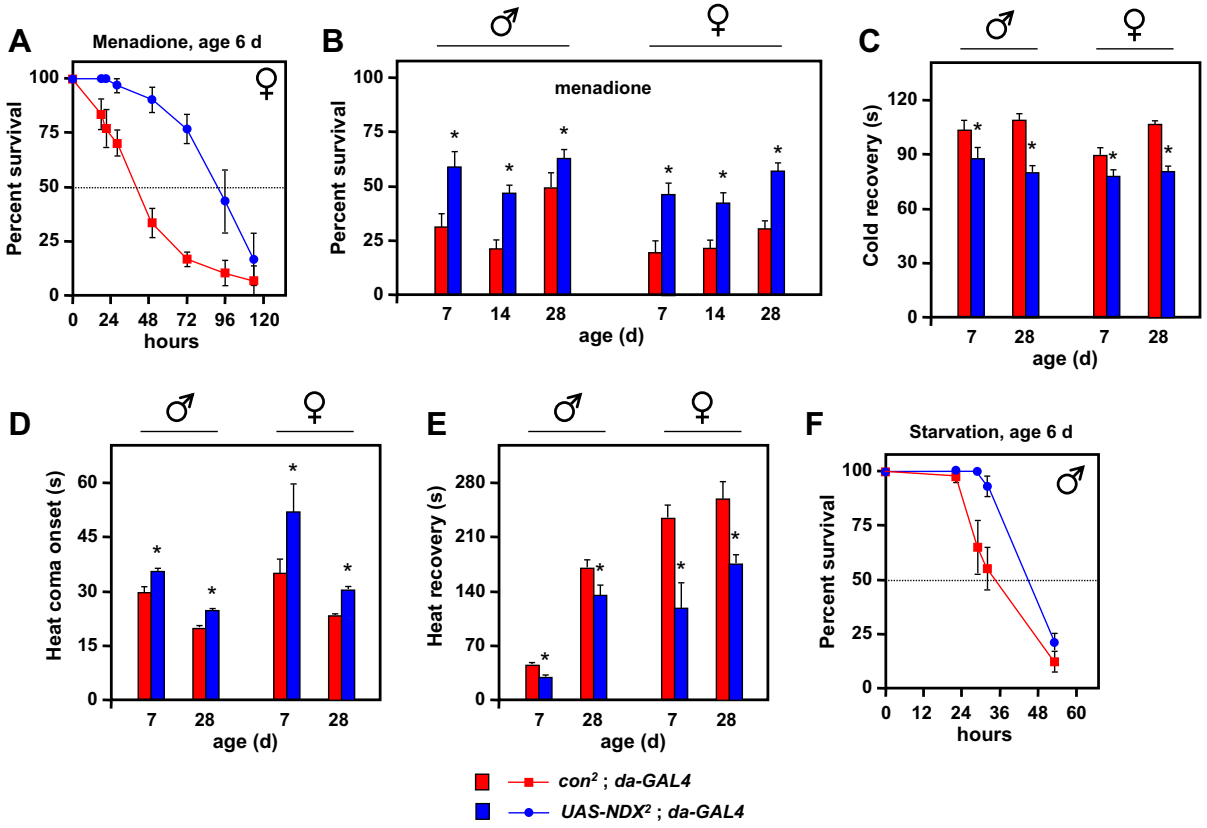

Fig. 4. Stress-resistance of NDX-expressing flies. (A) Survival kinetics and (B) survival to the 36 h time-point, of flies of the age, sex and genotype as indicated, in presence of menadione. Means ± SEM, six independent replicate experiments. (C, D, E) Times of recovery from, or onset of, thermally-induced coma, for flies of the age, sex and genotype as indicated. Means ± SEM, for groups of 8–9 females or 20 males. (F) Survival kinetics under starvation, of flies as indicated. In panels (B–E), all values for NDX-expressing flies were significantly different from those for control flies of the same age and sex (t test, $P < 0.05$), as indicated by the asterisks.

makes no presumptions on these points. Further submitochondrial isolation experiments could be done in combination with protease digests to address the localization question in more detail, in a future experiment.

4.2. Structure and predicted interactions of NDX

Modeling of NDX based on the structure and catalytic mechanism of yeast Ndi1 [42,43] reveals a compact structure with conservation of amino acids important for substrate binding. These include the first FAD-binding Rossmann fold (Fig. 6), where Trp-63 and Gly-64 (Ndi1 numbering) are responsible for binding of the FAD pyrophosphate moiety [43]. The adenine-binding Ser-61 is replaced by Thr as in many fungi and plants, whilst residues interacting with the isoalloxazine ring (Ala-393 and Gln-394) and the ribose moiety (Arg-85 and Val-176), plus those forming the hydrophobic channel (Phe-90, Leu-444, Leu-447, and Tyr-482) are conserved. The ubiquinone-binding site is also similar, with Gly-408 corresponding to Gly-445 of Ndi1, forming a hydrogen bond with the ubiquinol hydroxyl group. These conserved features are consistent with NDX having a similar function and substrate specificity as Ndi1. NDX lacks any calcium-binding EF-hand motif, such as those seen in many externally oriented alternative NADH dehydrogenases of plants and fungi [6,7], and which has been

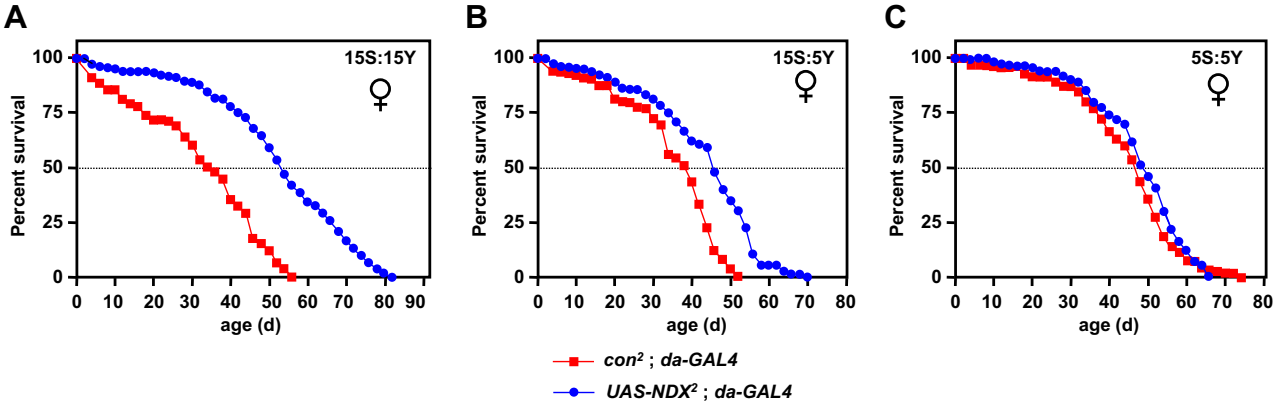

Fig. 5. Lifespan curves for NDX-expressing flies. Curves plotted for females of the indicated genotypes, on different media: (A) 15% sucrose (w/v), 15% yeast (w/v), denoted 15S:15Y, (B) 15% sucrose, 5% yeast (15S:5Y), (C) 5% sucrose, 5% yeast (5S:5Y). See also Fig. S3.

Table 1
Test for NDX complementation of cI knockdown.

Cross[a]	CG3683-KD female	CG3683-KD male	CG6020-KD female	CG6020-KD male
NDX	61/463 (13%)	60/367 (16%)	50/327 (15%)	55/484 (11%)
Empty vector	0/268 (0%)	2/387 (1%)	40/395 (10%)	31/561 (6%)
Significance[b]	$P < 0.0001$	$P < 0.0001$	$P < 0.01$	$P < 0.0001$

[a] In each cross, both parents were homozygous for *either* NDX or the empty vector, as indicated, on different chromosomes. All progeny therefore carried two hemizygous copies of either NDX or the empty vector. Reciprocal crosses used flies carrying knockdown constructs targeted against the indicated genes, over a CyO balancer chromosome, mated to flies homozygous for da-GAL4. Rescued progeny were therefore those lacking the CyO marker. In each case are quoted the absolute numbers of rescued (versus CyO control) flies eclosing, pooled from at least 2, mostly 3 experiments, with percentages shown in parentheses.

[b] Based on chi-squared test, the numbers of progeny bearing NDX were in every case significantly above expectation, based on the number carrying only empty vector.

suggested to confer the ability to use NADPH as substrate [7], although this is also disputed. Intriguingly, it also contains six cysteine residues whereas Ndi1 contains none, although two are seen in many plant and fungal NADH dehydrogenases (Fig. 6), and two others are widespread in metazoans.

The molecular weight of mature NDX, based on Western blots of both the epitope-tagged and native versions of the protein, is consistent with cleavage near the site predicted by Mitoprot (after residue 50), implying that NDX is imported and processed by the canonical pathway for mitochondrially targeted proteins. The mature protein would retain all functionally conserved elements of the single-subunit NADH dehydrogenases.

4.3. Phenotypic effects of NDX expression in Drosophila

Regarding those phenotypic parameters tested in both cases, the expression of NDX conferred similar properties on *Drosophila* as Ndi1. Both transgenes extend lifespan, although NDX had a much weaker relative effect under conditions of dietary restriction, whereas lifespan-extension by Ndi1 was independent thereof [9]. However this finding should be treated with caution, since the genetic backgrounds used were different, even though each employed an isogenetic control.

Two copies of NDX, combined with two copies of the *da-GAL4* driver, were required to convincingly reveal both enzymatic activity and partial rescue of developmental lethality caused by cI knockdown. The latter was also temperature-dependent, being ineffective at 25 °C. Thus, NDX appears to have a weaker phenotypic effect than Ndi1. The temperature-difference in complementation efficiency may be due to the fact that *C. intestinalis* has a lower optimal temperature in its natural environment than *S. cerevisiae* [44–46]. However, NDX expression in *Drosophila* had a protective effect from both heat- and cold-stress, suggesting that it is less heat-labile than the fly's own cI. An effect of insertion site on transgene function can also not be excluded, since NDX used a standard recipient strain with an engineered integration site, whereas Ndi1 and AOX transgenic lines were produced using random, P-element mediated integration. However, AOX lines created subsequently by targeted integration [A. Andjelkovic, unpublished data] retain full activity. One of the integration sites used in the present study (VK00001 site on chromosome 2) may be subject to position effects [40], although this site has recently been used for functionally successful insertion of a specific *GAL4* driver [47]. Moreover, integration on chromosome 3 produced a similar amount of functionality (data not shown). One possibility that should be considered is that NDX is stabilized only under stress conditions where its activity is required, such as known components of the alternative respiratory chain in plants [48–50]. However, this would require that the signals bringing about any such response be intrinsic to the protein or that any extrinsic molecular machinery that is involved must be conserved in *Drosophila*, despite the absence of NDX as a specific substrate since at least the emergence of the arthropods. In its natural context in *Ciona*, the gene is intronless, an organization common amongst stress-inducible genes [51].

The weak rescue of cI knockdown produced by NDX might reflect the fact that NDX co-exists naturally with cI, and might be stabilized or functionally activated via a structural interaction with it, that is

Fig. 6. Amino-acid sequence conservation of NADH dehydrogenases from different taxa. Ci — *Ciona intestinalis*, Sc — *Saccharomyces cerevisiae* Ndi1, Um — *Ustilago maydis* (XP_757559.1), An — *Aspergillus niger* (XP_001392541.2), Sm — *Selaginella moellendorffii* (XP_002970203.1), Sb — *Sorghum bicolor* (XP_002456580.1), St — *Solanum tuberosum* (NDB1, CAB52797.1), Nv — *Nematostella vectensis*, Ct — *Capitella teleta*, Cg — *Crassostrea gigas*, Ac — *Aplysia californica*. (A) Rossmann fold motif, with conserved residues mentioned in text highlighted in yellow (equivalent to Ser-61, Trp-63 and Gly-64 of Sc Ndi1), and showing the immediately adjacent targeting presequence of Ci NDX. (B) The sequences surrounding the six cysteines of Ci NDX, highlighted in orange, aligned with the corresponding regions from the other species. The numbering shown is of the Ci sequence.

conserved between *Drosophila* and *Ciona*. The knockdown of the subunits tested, CG6020 (NDUFA9) and CG3683 (NDUFA8), results in a substantial decrease in the amount of assembled cI [9]. In contrast, NDX was active when cI was inhibited by rotenone, which has not been reported to disturb the overall cI structure. However, since no structural interaction between cI and single-subunit NADH dehydrogenases has previously been reported, alternative explanations, such as consequent alterations in membrane structure, composition or fluidity, should also be considered. The difference in complementation efficiency between yeast Ndi1 and *Ciona* NDX might also reflect differences in substrate affinity, and that when cI is unavailable, other mechanisms limit the level to which intramitochondrial NADH can accumulate. Lastly, there may be functions of cI other than NADH oxidation, for which Ndi1 but not NDX can substitute.

NDX also protected against menadione toxicity. Menadione is considered to be a ROS (superoxide) generator, exerting its major toxic effect inside mitochondria, where enzymes containing oxidatively vulnerable Fe–S clusters (including aconitase and complexes II and III, as well as cI) are essential for normal metabolism. NDX expression conferred prolonged survival time in the presence of menadione, but flies still succumbed eventually, suggesting either that cI is the most susceptible, but not the only target of the toxin, or that NDX is unable to replace the full function of cI, which is in any case clear from the fact that it is non-proton-motive. An alternative explanation for the changes seen in stress resistance and in lifespan would be a hormetic effect, whereby mitochondrial impairment or damage produced by NDX induces a more robust stress response. Expressing a catalytically-inactivated mutant should distinguish between these possibilities.

4.4. Conclusions

The degree to which NDX will prove useful therapeutically remains to be determined. Although its phenotypic effects in *Drosophila* appear to be more modest than those of Ndi1, at least in regard to the complementation of cI deficiency, the maximal rate of substrate oxidation in organello in the presence of the cI inhibitor rotenone was similar to the maximal rate of substrate oxidation in organello conferred by *C. intestinalis* AOX in the presence of the cIV inhibitor cyanide [25], i.e. approximately 20% of the uninhibited rate.

If the hypothesized interaction between NDX and cI applies, pathologies associated with the structural absence of cI may not be ameliorated by implementation of NDX, whereas those in which cI is only functionally impaired, for example due to ROS-induced damage, may be curable. An obligatory association of NDX with cI and/or cIII would be logical, if much of cI in vivo is organized in supercomplexes to facilitate efficient channeling of electrons and to limit reverse electron flow. NDX activation would thus be calibrated to the activity of cI, and would only be brought into service when cI is overloaded, damaged or inhibited. Future experiments should aim to test this proposition and, if supported, its physiological consequences, as well as addressing the alternate explanations suggested above.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.08.001.

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