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The alternative enzymes-bearing tunicates lack multiple widely distributed genes coding for peripheral OXPHOS subunits

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ABSTRACT

The respiratory chain alternative enzymes (AEs) NDX and AOX from the tunicate *Ciona intestinalis* (Ascidiacea) have been xenotopically expressed and characterized in human cells in culture and in the model organisms *Drosophila melanogaster* and mouse, with the purpose of developing bypass therapies to combat mitochondrial diseases in human patients with defective complexes I and III/IV, respectively. The fact that the genes coding for NDX and AOX have been lost from genomes of evolutionarily successful animal groups, such as vertebrates and insects, led us to investigate if the composition of the respiratory chain of *Ciona* and other tunicates differs significantly from that of humans and *Drosophila*, to accommodate the natural presence of AEs. We have failed to identify in tunicate genomes fifteen orthologous genes that code for subunits of the respiratory chain complexes; all of these putatively missing subunits are peripheral to complexes I, III and IV in mammals, and many are important for complex-complex interaction in supercomplexes (SCs), such as NDUFA11, UQCR11 and COX7A. Modeling of all respiratory chain subunit polypeptides of *Ciona* indicates significant structural divergence that is consistent with the lack of these fifteen clear orthologous subunits. We also provide evidence using *Ciona* AOX expressed in *Drosophila* that this AE cannot access the coenzyme Q pool reduced by complex I, but it is readily available to oxidize coenzyme Q molecules reduced by glycerophosphate oxidase, a mitochondrial inner membrane-bound dehydrogenase that is not involved in SCs. Altogether, our results suggest that *Ciona* AEs might have evolved in a mitochondrial inner membrane environment much different from that of mammals and insects, possibly without SCs; this correlates with the preferential functional interaction between these AEs and non-SC dehydrogenases in heterologous mammalian and insect systems. We discuss the implications of these findings for the applicability of *Ciona* AEs in human bypass therapies and for our understanding of the evolution of animal respiratory chain.

1. Introduction

The process of oxidative phosphorylation (OXPHOS) uses the energy of nutrient oxidation for bulk production of adenosine triphosphate (ATP) by the mitochondria. Coenzyme Q (CoQ) reduction in the mitochondrial inner membrane is the first step in this process, and may be catalyzed by several different enzymes, such as nicotinamide adenine dinucleotide (NADH) dehydrogenase (complex I, CI), succinate dehydrogenase (complex II, CII), mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), electron transfer flavoprotein-ubiquinone oxidoreductase, among others. In humans, other vertebrates and insects, CoQ reoxidation is accomplished by the action of CoQ-cytochrome *c* oxidoreductase (complex III, CIII), which transfer the electrons to cytochrome *c*. This is in turn reoxidized by cytochrome *c* oxidase (complex IV, CIV) at the same time that O_2 is reduced to water. ATP synthase can then utilize the proton gradient created across the inner membrane during electron transport by CI, III and IV to phosphorylate ADP as the protons return to the mitochondrial matrix [1].

In addition to establishing the protonmotive force for ATP synthesis, the electron transfer reactions sequentially catalyzed by CI, III and IV

Abbreviations: AOX, alternative oxidase; NDX, alternative NADH dehydrogenase; SC, supercomplex; OXPHOS, oxidative phosphorylation; ATP, adenosine triphosphate; CoQ, Coenzyme Q; NADH, nicotinamide adenine dinucleotide; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; mGPDH, mitochondrial glycerol-3-phosphate dehydrogenase; TCA, tricarboxylic acid; AE, alternative enzyme; ROS, reactive oxygen species; PMT, putatively missing in tunicates; RMSD, root mean square deviation; PG, propyl-gallate; AA, antimycin A; Ldh, lactate dehydrogenase.

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have other unique features. CI is the only inner membrane dehydrogenase in vertebrates and insects that reduces CoQ and reoxidizes the mitochondrial pool of the diffusible electron carrier NADH, providing NAD^+ molecules that are necessary for the tricarboxylic acid (TCA) cycle, the pyruvate dehydrogenase complex, the β-oxidation of fatty acids, and other pathways. The other CoQ-reducing enzymes are usually FAD-linked, associated with more specific pathways, and not proton-pumping. CI, III and IV can also interact physically to each other to form supramolecular assemblies generally termed as supercomplexes (SCs) (reviewed in [2,3]). The SC CI/III_2/IV is also referred to as the respirasome and is apparently how the great majority of CI is arranged in mammalian mitochondria [4,5], which arguably helps streamline electron transfer from NADH to O_2 [6] and/or maintains the structural stability and function of each individual complex [7,8]. Along with oligomers of ATP synthase, SCs have also been implicated in stabilizing the mitochondrial cristae [9], helping the organelle to maintain its characteristic appearance. Although CII may participate in SCs in ciliate eukaryotes [10], this does not appear to be the case in mammals, nor any other CoQ-reducing enzyme has been reported as part of SCs to date.

The unique nature of CI and CIII/IV in being, respectively, the only sites for CoQ reduction by NADH and CoQ reoxidation by O_2 in human mitochondria, makes the respiratory chain vulnerable to dysfunction (caused by mutations or chemical inhibition) that may lead to mitochondrial diseases. Interestingly, most organisms (including most animal groups, but not vertebrates and insects) have additional, structurally simpler, single-subunit enzymes that can also catalyze similarly the CoQ reduction and reoxidation reactions described above, but without proton pumping. These alternative enzymes (AEs) may provide metabolic flexibility by branching out the respiratory chain and maintaining metabolic flux when CI and/or CIII/IV are blocked/unfunctional, even if this may affect the efficiency of ATP production. Several fungi species that naturally produce OXPHOS inhibitors to potentially outcompete AE-nonbearing species for local resources, utilize their AEs as alternative ways to maintain O_2 consumption and the mitochondrial levels of NAD^+, bypassing their own CI and/or CIII/IV [11,12]. In the cases of the plant species *Symplocarpus renifolius* (Magnoliophyta: Araceae) and *Nelumbo nucifera* (Magnoliophyta: Nelumbonaceae), the AEs are highly upregulated in the flowering parts to function deliberately in detriment of ATP production, as thermogenic enzymes that uncouple mitochondria for heat dissipation [13,14], analogously to UCP1 in brown adipose tissue of mammals [15].

The roles of AEs in animals are still much more obscure, given that it was assumed for a long time that these enzymes were not present in Metazoa. They are in fact absent from vertebrates, insects and other groups, but it appears that most animal taxa have at least one copy of an AE coding gene in their genome [16–19], so AEs must be important for these species. The best studied animal AEs are the alternative NADH dehydrogenase NDX and the alternative oxidase AOX from *Ciona intestinalis*, an ascidian (Chordata: Tunicata) that has been used as animal model in ecology of invasive marine species and in chordate embryology [20,21]. However, the context in which they have been studied is of biomedical application as potential therapies for human mitochondrial diseases. Since NDX can bypass CI function, it could theoretically help to maintain the flow of electrons from NADH to the respiratory chain in cases of genetic mutations affecting any of the numerous subunits and assembly factors of CI. Similarly, by transferring electrons directly from reduced CoQ to O_2 and thus bypassing CIII/IV, AOX could aid in reoxidizing CoQ upon defects in any of these two complexes. These ideas have been tested extensively in different human cell culture, mouse and fly models, which naturally do not possess AEs, with diverse outcomes. In general, as expected, *Ciona* NDX and AOX respectively promote in these models respiration resistant to the CI inhibitor rotenone and to the CIII/IV inhibitors antimycin A and cyanide, preventing excessive production of reactive oxygen species (ROS) [22–26]. When xenotopically expressed in the insect *Drosophila melanogaster*, they confer resistance to several types of organismal stress that directly or indirectly affect mitochondrial function, such as exposure to ROS generators or other toxicants, and thermal stresses [23,24,27,28]. NDX, but not AOX, extends fly lifespan [24,27,29] presumably by a mechanism of reverse electron transport to CI, similarly to what has been described for the expression of the yeast NADH dehydrogenase Ndi1 [30]. NDX can partially rescue the developmental arrest in flies caused by the knockdown of CI subunits [27], and AOX is able to genetically complement, at least partly, a range of CIII/IV defects in cells, mice and flies [23,31–34], and to ameliorate other mitochondrial and non-mitochondrial dysfunction [35–37]. In contrast, AOX expression appears to be neutral in fly models of mitochondrial DNA replication and protein synthesis defects [38,39], and very detrimental in a mouse model of CIV-induced myopathy due to its antioxidant properties [40]. AOX-expressing flies also have problems in producing mature sperm cells [41], and are very dependent on a high-nutrient diet for successful development [28,42].

These contrasting effects of NDX and AOX in naturally AE-free systems may reflect metabolic and/or structural features of the mitochondrial environment of *Ciona* and other AE-bearing animals, which might be different than that of AE-nonbearing species such as vertebrates and insects. The independent loss of AE-coding genes in the latter groups (and therefore the retaining of AEs in tunicates and other animal groups) might have been in itself an impactful evolutionary event that changed their mitochondria significantly. Here, we sought to compare and contrast the composition of the respiratory chain of humans/mammals and *Drosophila* (AE-nonbearing representatives) with that of the AE-bearing *Ciona*, using a combination of genomic, structural modeling and biochemical data. We report that the *Ciona* genome lacks clear orthologues of several genes encoding peripheral subunits of CI, III and IV, some of which are essential for SC formation in mammals. We also show that *Ciona* AOX cannot access all CoQ molecules reduced by CI, but it is readily available to oxidize CoQ molecules reduced by the non-SC dehydrogenase mGPDH, a feature that might reflect how this AE functions in the mitochondrial inner membrane of tunicates.

2. Results

2.1. Putatively missing OXPHOS genes and the modeling of *Ciona* respiratory chain

To get insight into the possible differences between the respiratory chains of AE-bearing and -nonbearing animals, we first attempted to retrieve the genomic sequences for all genes encoding subunits of OXPHOS CI-IV and the ATP synthase from *C. intestinalis* and its closest relatives, *C. robusta* and *C. savignyi*, based on orthologous sequences from humans and *D. melanogaster* (Supp. Table S1). Supp. Table S2 shows the accession numbers and/or genomic positions of the identified orthologues, and the prediction values of the mitochondrial target sequence of their encoded polypeptides. Curiously, we consistently failed to identify specific gene sequences for several CI, III and IV subunits and for the ATP synthase subunit ATP5MJ (Supp. Tables S1 and S3), despite using relaxed tBLASTn search parameters, BLASTp with the PSI-BLAST algorithm, and more sensitive HMM-based approaches. We expected not to identify orthologous genes for ATP5MJ, the CI subunit NDUFC1, and the CIV subunit COX7A2L, as they have been described as vertebrate-specific subunits [2,43–45], but not finding additional thirteen OXPHOS-coding genes was initially surprising. We then expanded our searches to other tunicate species and observed that most of these putatively missing genes do not appear to be present in any tunicate genome available in the GenBank, Aniseed and Ensembl databases and in any reference proteome in the UniProtKB and Swiss-Prot databases (Supp. Table S3), suggesting that their encoded polypeptides are not part of tunicate CI, III and IV, or that they have evolved so rapidly that are now undetectable by the current approaches of sequence similarity searches. Hereafter, we refer to these genes/subunits as PMT (putatively missing in tunicates).

Next, we took advantage of the many atomic structures available in the Protein Data Bank (www.rcsb.org) for the mammalian CI, III and IV, and mapped the location of the PMT subunits to test if their putative absence is supported from a structural point-of-view. Except for CI NDUFV3, all other PMT subunits are membrane proteins, located on the periphery of the three complexes (Fig. 1A, C and D). CI and CIII have nine and two PMTs, respectively, many of which in mammals interact with membrane lipids, such as cardiolipin, phosphatidylcholine and phosphatidylethanolamine (Fig. 1). More importantly, almost all membrane PMT subunits of CI interact physically with each other on the intermembrane side, forming a kind of network that appears to provide structural support for the CI membrane arm (Fig. 1A and B).

We also modeled the structures of the OXPHOS subunits encoded by all identified *C. robusta* genes shown in Supp. Table S1 using I-Tasser [46] and AlphaFold2 [47,48], and explored the regions of protein-protein interactions between the PMT and the identified subunits in the mammalian CI, III and IV structures. We initially used the high root mean square deviation (RMSD) values of alignments between the *Ciona* models and the mammalian structures (Supp. Table S4) as indication of significant structural changes that could accommodate the putative lack of interacting subunits. The models of the *Ciona* CI subunits ND3, ND4L and ND6, for example, aligned with their mammalian counterparts with high RMSD, respectively, although all α-helices, loops and general architecture of the polypeptides are easily recognizable (Fig. 2A). All these subunits in mammals directly interact with the CI subunits NDUFA1, NDUFA3 and NDUFS5 (Fig. 2B), which are PMTs. The major difference between the models and the atomic structures is in the loop that connects α-helices 4 and 5 of ND6 (herein referred to as loop4-5), which is quite variable between tunicates and other animals, and much shorter in *Ciona* (Fig. 2A, B and Supp Fig. S1). In mammals, ND6 loop4-5 and the N-terminal end of α-helix 5, along with ND3 loop2-3 and ND4L loop2-3, form a hub of interactions with NDUFA1, NDUFA3 and NDUFS5, which in turn are interconnected with almost all other PMT subunits of the CI membrane arm.

We also identified sequence and structural divergence between *Ciona* models and mammalian subunits even when their alignments had low RMSD values. The interactions of CI subunits ND2, ND4, ND5 and NDUFB4 with the PMT subunit NDUFA11 in the mammalian CI structure appears to be largely mediated through four lipid molecules: two phosphatidylethanolamines, a phosphatidylcholine and a cardiolipin (Supp. Fig. S2A). However, the final eight C-terminal residues of NDUFA11 interact with the C-terminus of α-helix 11, the N-terminus of α-helix 12 and the loop between them (loop11-12) in the mammalian structure of ND2 (Supp. Fig. S2B—C), a region that is also shorter and divergent in *Ciona* species (Supp. Figs. S2C and S3). The C-terminus of NDUFA11 α-helix 2 also interact with the C-terminal region of ND5 (Supp. Fig. S4A), which forms a long extension protruding from the subunit central core (Supp. Fig. S4B). The ND5 C-terminus is the only region with significant structural divergency in the *Ciona* models, compared to the atomic structure of the mammalian orthologue (Supp. Fig. S4B and S5).

For CIII, the two PMT subunits also interact extensively with each other and with membrane lipids, as if these subunits were to hold the lipids in place on the periphery of each monomer in the dimeric CIII structure (Fig. 3A). The N-terminus of the PMT subunit UQCR11 interacts with the extreme C-terminus of UQCRB in mammals (Fig. 3B), but in tunicates the UQCRB C-terminus is >40 amino acids longer (Fig. 3C and Supp. Fig. S6), with no identifiable homology with any animal sequence. AlphaFold2 was able to model a curved α-helix in this region (Supp. Fig. S7). It is unlikely that this extended C-terminal region

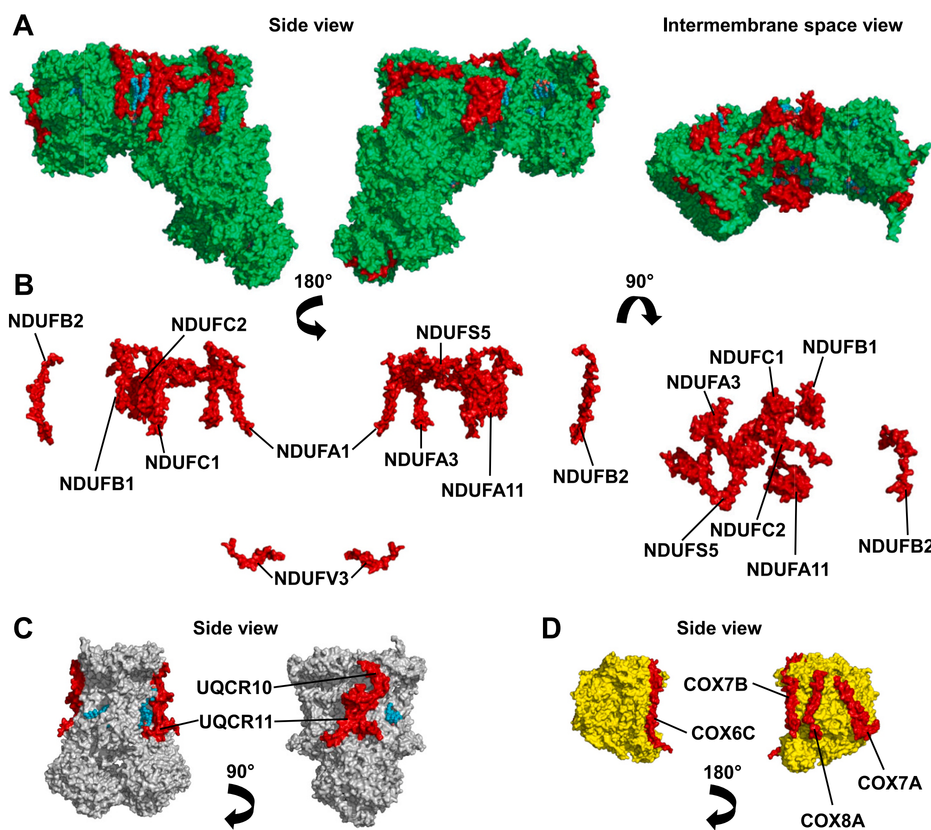

Fig. 1. Putatively missing respiratory chain subunits in *Ciona*. The subunits of complexes I (A, B), III (C) and IV (D), whose encoding genes are not found in the genome of tunicates, are shown in red, as they appear in the mammalian structure deposited in PDB under accession number 5XTH. Complex I subunits that are found in tunicates have been removed in B to highlight the interconnection among almost all putatively missing complex I subunits in *Ciona* (see text for details). Membrane lipids are shown in cyan. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

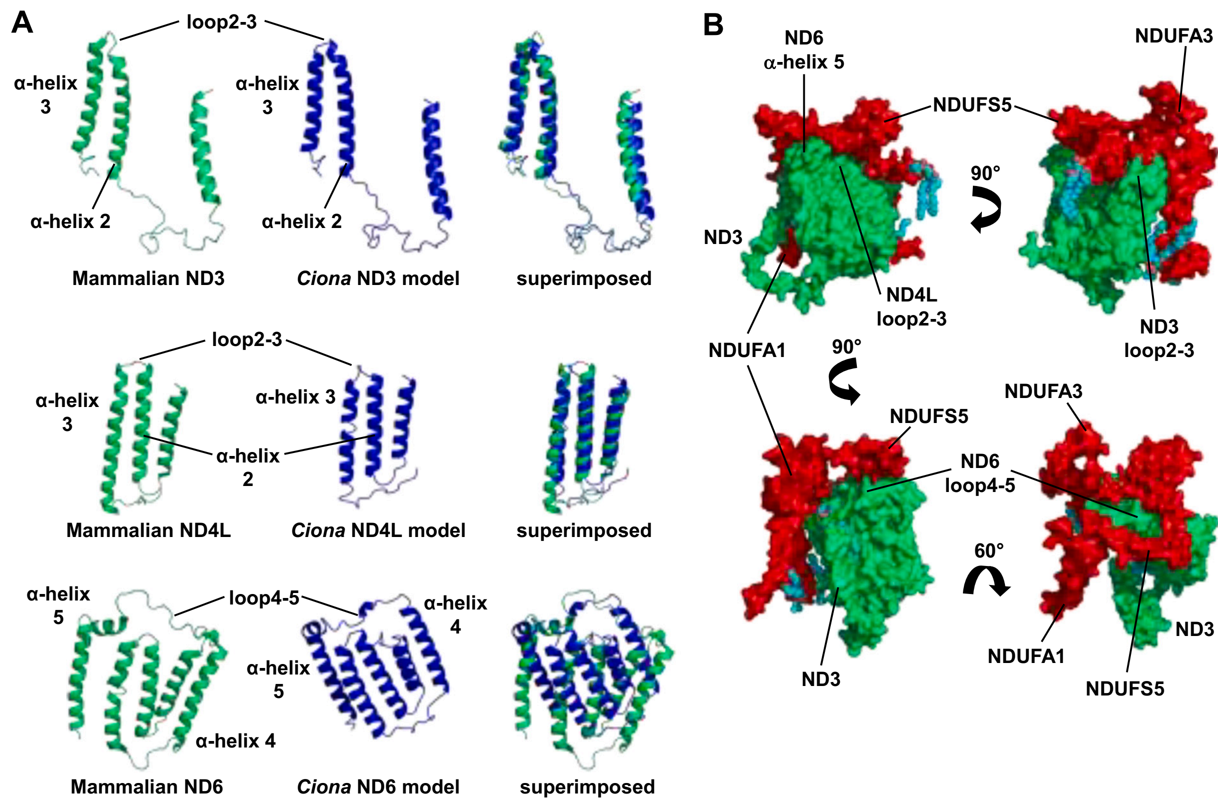

Fig. 2. Structural divergency between mammalian and *Ciona* ND3, 4 L and 6. A, Structural alignments between the mammalian atomic structure and the I-Tasser-generated *Ciona* models of the indicated complex I subunits. The AlphaFold2-generated models are congruent and not shown for clarity purposes. RMSD values are shown in Supp. Table S4. B, Details of the interactions among ND3 loop2-3, ND4L loop2-3 and ND6 loop4-5, with the putatively missing subunits NDUFA1, NDUFA3 and NDUFS5, as shown in the mammalian structure deposited in PDB under accession number 5XTH.

is encoded by another gene located downstream of the UQCRB gene in the *Ciona* genome and was mistakenly assigned as part of the polypeptide, as the C-terminal extension coding sequence is found in frame with the codons for all other amino acids in the only exon of the UQCRB gene, and in mature transcript sequences deposited in the GenBank (data not shown).

In the case of CIV, none of the PMT subunits interact with each other or with membrane lipids. We identified a significant discrepancy between the I-Tasser- and AlphaFold2-generated models of *Ciona* COX7C (Supp. Fig. S8A). The mammalian COX7C C-terminus is a long α-helix that makes extensive hydrophobic contacts with COX8A, which is a PMT (Supp. Fig. S8B). Amino acid sequence alignment shows that this region in *Ciona* COX7C is not too divergent among species, but secondary structure prediction shows low probability for a long α-helix (Supp. Fig. S9). Whereas AlphaFold2 recovered the full extension of this C-terminal α-helix, I-Tasser was unable to do so (Supp. Fig. S8A). The divergence in sequence and predicted structure in the regions of *Ciona* subunits pointed out above correlates with the prospect that a significant number of genes encoding OXPHOS subunits appear to have been lost from tunicate genomes. This might have significant impact on the function of the tunicate respiratory chain, especially in the context of AEs, as we show below.

2.2. Putatively missing OXPHOS gene products, supercomplex formation and alternative enzymes

Although we identified a total of fifteen PMT genes for OXPHOS subunits, none of these have been reported involved directly in electron transport or proton pumping by the mammalian CI, III and IV, i.e., none are core subunits of these complexes. We were also unable to identify by modeling structural features of the remaining subunits that could indicate alterations in these essential activities of the three complexes. Because all PMT subunits are peripherally located in their own complex, we then analyzed if they could be involved in the formation of SCs. Using the structures of the porcine and ovine CI/CIII$_2$/CIV SCs [49,50], we observed that some of the PMT subunits are in fact key elements in mediating complex-complex interactions (Fig. 4). The PMT subunit NDUFA11 of CI interact with CIII subunit UQCRQ in all structures, and the PMT subunit COX7A of CIV interact with the PMT subunit UQCR11 of CIII in the porcine structure [50] and with the CI subunit ND5 in one of the ovine SCs [49]. A paralogue of COX7A in mammals, COX7A2L (specifically its long isoform), which is also absent in tunicates, has been shown to stabilize the III$_2$/IV interactions either in the CI/III$_2$/IV or the III$_2$/IV assemblies [51,52].

Our data suggests that the molecular environment of the mitochondrial inner membrane in which *Ciona* AEs have evolved and function might be naturally free from SCs, or that *Ciona* SCs are assembled via a mechanism different from that of mammals, since we have failed to find many of the subunits important for complex-complex interactions in this organism. In the absence of *Ciona* samples to directly test the lack of SC hypothesis, we turned to biochemistry and transgenic flies to test if the function of AEs may be limited by the presence of SCs. *Drosophila* (and all insects, as far as we can identify from homology searches) has the same OXPHOS subunits as in mammals, except for CI subunit NDUFC1, CIV subunit COX7A2L, and ATP synthase ATP5MJ (Supp. Table S1), and its CI structure resembles that of mammals [53]. Importantly, SCs are also detected in *Drosophila* mitochondrial preparations [54–58], so it is plausible to assume this organism can help us address the question of how AEs interact functionally with SCs.

Drosophila has two main mitochondrial inner membrane-bound dehydrogenases that significantly contribute to mitochondrial respiration and ATP production: CI, which in mammals is mostly found in SCs [4,5],

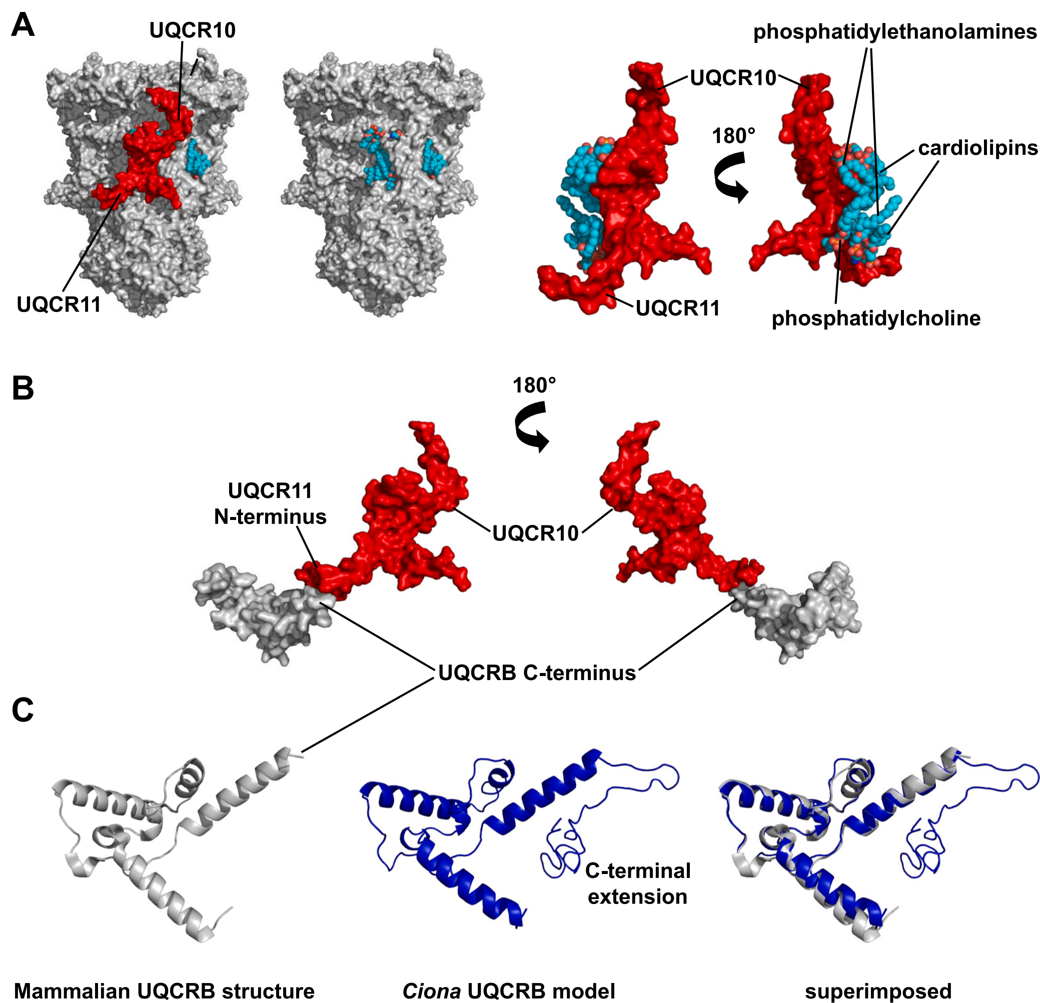

Fig. 3. Structural divergency between mammalian and *Ciona* UQCRB. A, Details of the general interactions among the mammalian UQCR10 and 11 with the dimeric CIII (shown in gray) and the indicated membrane lipids, as shown in the mammalian structure deposited in PDB under accession number 5XTH. B, Details of the specific interaction between UQCR11 and UQCRB. C, Structural alignment between the mammalian atomic structure and the I-Tasser-generated *Ciona* model of UQCRB (RMSD value shown in Supp. Table S4). See Supp. Fig. S7 for the AlphaFold2-generated model and text for more information.

and mGPDH, also known as glycerophosphate oxidase, for which to our knowledge no evidence of SC participation exists. In addition, *Ciona* AOX-expressing fly models are well characterized [23,33,59]. Our hypothesis was that, using *Ciona* AOX as a model for AE function, we would be able to detect that it preferentially reoxidizes CoQ that was initially reduced by mGPDH, "outside" SCs. We then measured mitochondrial oxygen consumption in control and transgenic AOX-expressing fly larvae at two mitochondrial states: 1) OXPHOS, in which the proton gradient generated by CI, III and IV is dissipated back to the mitochondrial matrix mainly by active ATP synthase, supporting a relatively high respiration; and 2) Leak, in which the ATP synthase is inhibited by oligomycin, supporting a relatively low respiration that is maintained by inherent proton leaks through the inner membrane. For each state, we evaluated separately CI- or mGPDH-driven respiration via addition of their respective oxidizable substrates, pyruvate/malate or glycerol-3-phosphate. We then examined the contribution of AOX to CI- or mGPDH-driven oxygen consumption by adding its inhibitor, propyl-gallate (PG). In a parallel assay, we evaluated CIII/IV contribution by inhibition with antimycin A (AA).

We observed that in the OXPHOS state, CI-driven respiration did not change in the presence of AOX (Fig. 5A, compare black bars in *iii* and *iv*). AA-resistant oxygen consumption, the hallmark of AOX activity, was ~1/3 of CI-driven respiration in AOX-expressing larvae (Fig. 5A, compare black and white bars within *iv*), comparable to previously published data [33]. As expected, no AA-resistant CI-driven respiration was detected in control flies (Fig. 5A, white bars in *iii*). In contrast, we observed that mGPDH-driven respiration in AOX-expressing larvae was ~30 % decreased compared to controls (Fig. 5A, compare black bars in *i* and *ii*). AOX also sustained ~1/3 of mGPDH-driven respiration in the presence of AA (Fig. 5A, compare black and white bars within *ii*), whereas no AA-resistant respiration was detected in control flies (Fig. 5A, white bars in *i*). We also measured transcript levels of *Gpo1*, the ubiquitously expressed mGPDH isoform in *Drosophila*, but no statistically significant changes were observed (Suppl. Fig. S10), indicating that the drop in mGPDH-driven respiration is regulated at the protein abundance and/or enzyme activity level. Transcript levels of the *AOX* transgenes were also confirmed in our fly lines (Suppl. Fig. S10).

In the Leak state, when mitochondrial membrane potential is maximal, oxygen consumption driven by either mGPDH or CI is increased in the presence of AOX (Fig. 5B, compare black bars in *v* and *vi*, and in *vii* and *viii*); AOX inhibition reverts this to control levels (Fig. 5B, compare black and gray bars within *vi*, and within *viii*). Inhibition of mGPDH-driven Leak respiration with AA in AOX-expressing flies leads to a ~ 60 % drop, while no respiration is detected in the controls, as expected (Fig. 5B, compare black and white bars within *v*, and within *vi*). The drop in mGPDH-driven Leak respiration after AOX inhibition with PG was ~40 % in AOX-expressing flies, and nonexistent in controls (Fig. 5B, compare black and gray bars within *v*, and within *vi*). The data

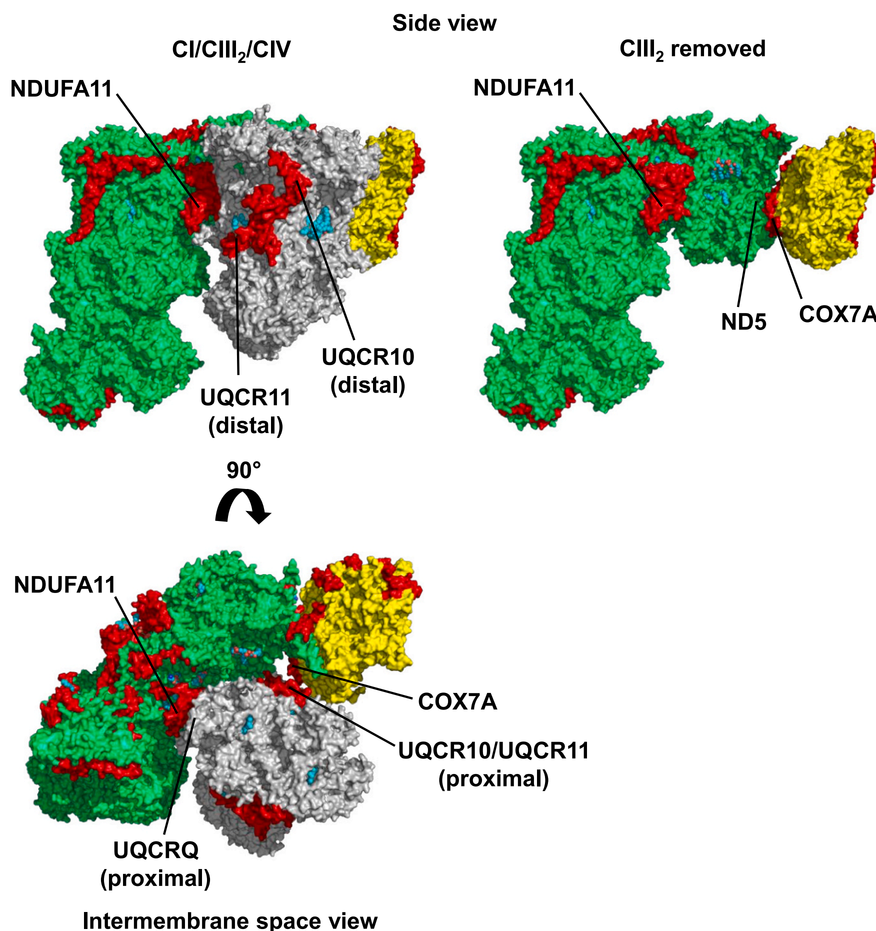

Fig. 4. Putatively missing subunits in *Ciona* are important for respiratory supercomplex formation in mammals. CI, dimeric III and IV are shown respectively in green, gray and yellow, according to the mammalian structure deposited in PDB under accession number 5XTH. All putatively missing subunits in *Ciona* are shown in red; the ones involved in complex-complex interactions are indicated (see text for details). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

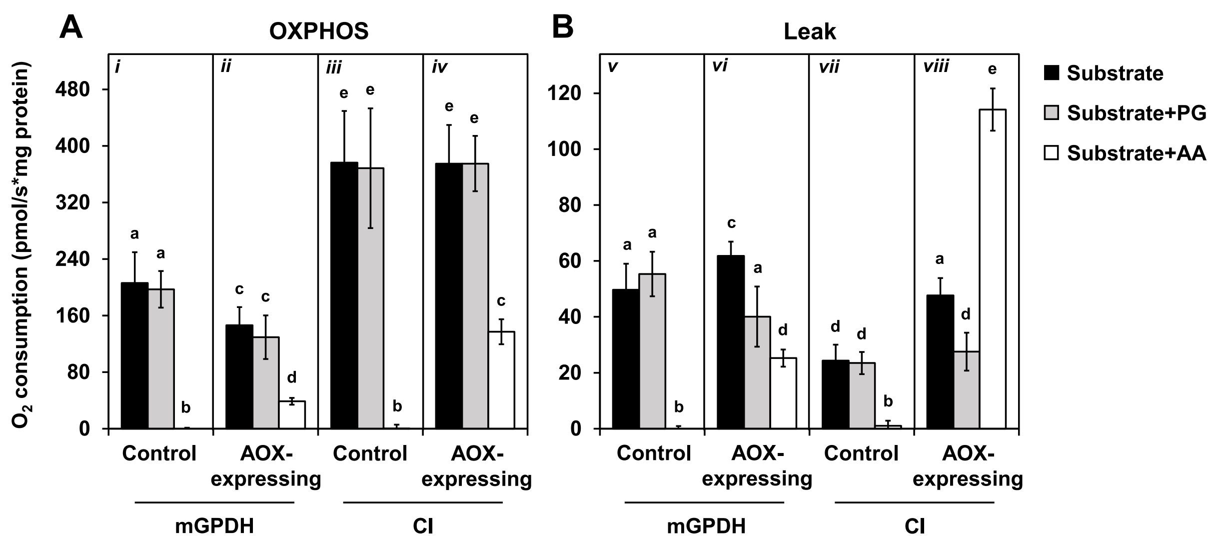

Fig. 5. Mitochondrial oxygen consumption by *Ciona* AOX is possibly restricted by CI-containing supercomplexes. The OXPHOS (A) and Leak (B) states were achieved, respectively, by ADP or olygomicin, after addition of substrates: glycerol-3-phosphate to activate the mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), or pyruvate/malate to activate complex I (CI). PG, AOX inhibitor propyl gallate; AA, CIII inhibitor antimycin A; control, w^{1118} larvae; AOX-expressing, 3x*tubAOX* larvae (see Materials and methods for details). The datapoints represent means ± standard deviations from 3 to 11 biological replicates. a-e indicate distinct statistical classes ($p < 0.05$), as determined by the Tukey's post hoc test following a one-way ANOVA analysis. The separation of the graphs in panels *i-viii* is meant to facilitate data interpretation (see Results for details).

is consistent with the idea that CoQ molecules reduced by mGPDH are being split for reoxidation to both AOX (~40 %) and CIII/IV (~60 %). In contrast, in AOX-expressing flies, CI-driven Leak oxygen consumption increased more than twofold when CIII/IV are inhibited (Fig. 5B, compare black and white bars within *viii*), reaching similar levels of the AA-resistant respiration obtained for the OXPHOS state (compare absolute values of white bars in Fig. 5A, *panel iv*, and in Fig. 5B, *panel viii*). This indicates that most CoQ molecules reduced by CI were not available for reoxidation by AOX when CIII/CIV were active; once AA was added, AOX then accessed these CI-reduced CoQ, and as a result, Leak respiration significantly increased. As shown previously [33], we confirmed that SCs are present in mitochondrial extracts of control and AOX-expressing flies via blue native-polyacrylamide gel electrophoresis (BN-PAGE, Supp. Fig. S11A). Together, our data is in agreement with the idea that CoQ molecules can be trapped in SCs, and that this leads to a preferential interaction between *Ciona* AOX and non-SC dehydrogenases in heterologous systems. Interestingly, treatment with OXPHOS inhibitors, such as the ones used in the mitochondrial respiration assays above, does not appear to disrupt *Drosophila* SCs, as judged by BN-PAGE analyses (Supp. Fig. S11B), suggesting that the increase in CI-driven Leak oxygen consumption supported by AOX upon CIII/IV inhibition occurs by simple diffusion of CoQ molecules from SCs.

3. Discussion

The presence of AE-coding genes in the animal kingdom was reliably reported only after the release and analyses of the first complete genomic sequences of animal species other than vertebrates and insects, two of the most diversified animal taxa on earth and which have been scientists' favorite organisms for centuries [16,17,19]. Finding AEs in sponges, placozoans, corals, tunicates, cephalochordates, hemichordates, echinoderms, mollusks, annelids and nematodes has established that the highly successful vertebrates and insects have lost the AE-coding genes independently early in the evolution of each of these taxa. In general, evolution of animal AEs (at least animal AOX) is apparently more complex, with a number of possible horizontal gene transfer events [18]. Nevertheless, two important questions can be raised about the distribution of AEs in animals [60]: are AEs important to be retained (or reacquired) in certain groups, and are they dispensable (or perhaps even detrimental) in others?

For long, the strongest hypothesis that could partially answer both questions came initially from results of the extensive research done with plant and fungi AEs that show that these are in general stress response enzymes (reviewed in [61,62]). Either biotic or abiotic stress can induce expression of AEs, often helping these organisms deal with the blockage of the canonical OXPHOS and with bursts of ROS, which may be part of an important defense strategy when the organisms are sessile, slow or have decreased mobile capacity and cannot simply flee to avoid harm. This appears to be also true for the sessile *C. intestinalis*, as AOX transcripts are upregulated in the presence of hydrogen sulfide [28,63]. This toxin can be produced by certain marine bacteria and accumulate in the local environment, inhibiting CIV of other organisms nearby [64]. In addition, AOX knockdown decreases sulfide tolerance by *Ciona* threefold [63]. Animals that could actively and rapidly move away from a sulfide-rich zone, for example, would face the problem of having decreased mitochondrial ATP production because of the energy-dissipating nature of AEs [60]. This trade-off might have played an important role in the selection processes that led to sessile and slow animals, such as tunicates and other AE-bearing species, to retain AEs to withstand dangerous environmental chemical exposures and other abiotic or biotic threats. Additionally, fast swimmers, runners and flying animals, which need tightly coupled mitochondria for efficient OXPHOS to sustain the muscle contractions essential for their mobility, might have lost AEs, coupling even more ATP synthesis and nutrient oxidation, and becoming more efficient at fleeing such dangerous conditions. Obviously, this hypothesis does not take into account the AEs being reacquired via lateral gene transfers, like it appears to be the case in species of Collembola and Sciaridae dipterans [18], animals which one would consider fast moving ones. In addition, it is important to state that the data on the roles of AOX in preventing toxic effects of sulfide in *Ciona* was obtained primarily during the larval phase, a developmental stage during which these animals actively swim. Therefore, losing, retaining or reacquiring AEs might not have had a single (or few) universal explanation(s); future case-by-case studies aiming at explaining the AE distribution in metazoans are thus warranted.

The reasons why CI, III and/or IV assemble in SCs are still debated. It has been proposed that these structures sequester their own dedicated CoQ and cytochrome *c* molecules, streamlining electron transfer from NADH to O_2 [65,66] and thus limiting ROS production [67,68]. However, this is not supported by structural, kinetic and spectroscopic analyses, which suggest free exchange of CoQ and cytochrome *c* between complexes in and out of SCs [3,49,69–72]. SCs may also have a role in stabilizing the individual complexes and ensuring their proper function [7,8]. A recent report has questioned these ideas by showing that disrupting SCs in mice ubiquitously, without affecting the levels of individual OXPHOS complexes, does not cause any significant detrimental phenotype nor it affects the function of individual complexes [73]. Our biochemical data from samples of AOX-expressing fly larvae (Fig. 5B) is consistent with the idea that SCs sequester CoQ and/or cytochrome *c* pools. *Ciona* AOX appears not to have access to this SC pool of reduced CoQ, unless electron transfer through CIII/IV is blocked by AA. *Drosophila* SCs appear to remain intact upon CIII/IV inhibition (Supp. Fig. S11B), suggesting that CoQ molecules can be reduced by CI in intact SCs and leave to be reoxidized by AOX. On the other hand, CoQ molecules reduced by the non-SC dehydrogenase mGPDH can be readily reoxidized by AOX. This is in agreement with a previous report showing that *Ciona* AOX xenotopically expressed in the mouse is also not engaged with CoQ molecules reduced by CI, unless an OXPHOS inhibitor is added [74]. Moreover, AOX in mice also enhances electron transport from another non-SC dehydrogenase, CII; the similar results we and others have obtained showing that *Ciona* AOX performs electron transport excluded from SCs agree with the possibility that the *Ciona* mitochondria might not have SCs, as judged by our inability to detect several OXPHOS orthologues in this organism. It is equally possible, though, that *Ciona* does form distinctly arranged SCs and that, in this environment, AOX has access to the SC-sequestered CoQ pool (see more discussion below). Even if tunicates form SCs by a mechanism different than that of mammals, it is still intriguing how important OXPHOS genes such as NDUFA11, UQCR11, COX7A, and other twelve not directly related to SC formation are missing (or have diverged so extensively beyond the point of detection) in these animals. In humans, several mutations in NDUFA1, NDUFC2, NDUFA11 and COX8A are associated with mitochondrial and neurodegenerative diseases ([75–79], the Mitochondrial Disease Sequence Data Resource Consortium, https://mseqdr.org/), and NDUFB2, UQCR10, UQCR11, COX7A1, COX7B and COX8C gene variants are linked to preneoplastic conditions, several types of cancer and other diseases ([80–82], the DisGeNET Gene-Disease Associations databank, https://www.disgenet.org). NDUFA11 knockdown in rat cardiac cells in culture significantly increases ROS levels, in addition to lowering SC formation and OXPHOS activity [83]. Moreover, ubiquitous COX6C and COX7A knockdown in flies is developmentally lethal [33,59,84], and skeletal muscle-specific deletion of COX7A1 in mice decreases OXPHOS activity and angiogenesis [85].

The bricolage hypothesis of cellular respiration evolution states that the canonical respiratory chain arose by combining and altering preexisting prokaryotic enzymes, and further evolved with the addition of extra subunits in eukaryotic mitochondria that supported more complex assembly, stability, and regulation, culminating in the functional and structural complexities seen in SCs (reviewed in [86]). SCs have been described in bacteria, archaea, yeast, plants, unicellular eukaryotes, insects and mammals [10,33,49,50,55,57,58,87–93], but many of the subunits involved in mammalian SC formation are not present in

prokaryotes, and if they participate in other eukaryotes' SC is yet to be shown. This diverse composition and arrangements of SCs found in living organisms may suggest that they evolved independently multiple times in different taxa and, therefore, have different assembly mechanisms [86]. However, the conservation of OXPHOS genes, subunit composition and SC types — even though in different relative abundances [58] — in mammals and flies would argue that most animals (at least the deuterostomes and protostomes) form SCs via similar mechanisms, and that the tunicate respiratory chain might have become structurally simpler. This correlates with the structural variability we found for the models of *Ciona* OXPHOS subunits that would interact with the PMT subunits (Figs. 2–3, Supp. Figs. S2, S4, S7 and S8), and with the fact that the *Ciona* mitochondrial genome, whose protein-coding genes all encode central OXPHOS subunits, has evolved much faster than in mammals and insects [94]. It is important to emphasize, however, that the putative structural features of the tunicate respiratory chain we are proposing here are based mainly on findings provided by the current homology detection tools available, the limitations of which may not allow the identification of highly divergent orthologues. All PMT subunits in *Ciona* are on the outside of other animal's CI, III and IV, often interacting with lipids in the inner membrane (Fig. 1–3, and Supp. Fig. S2) and likely subjected to different evolutionary pressures. The *Drosophila* COX7B (the product of the *CG7630* gene) is an example of a "found" OXPHOS subunit with similar characteristics and very few conserved amino acid residues with mammals. It was only firmly placed as a fly CIV subunit recently, after extensive genetic, biochemical and physical data were obtained [95]. Studying tunicate respiratory chain extensively at the structural and functional level will thus be instrumental to determine SC presence and to understand its roles in animals.

Assuming our failure to identify fifteen OXPHOS genes in tunicates

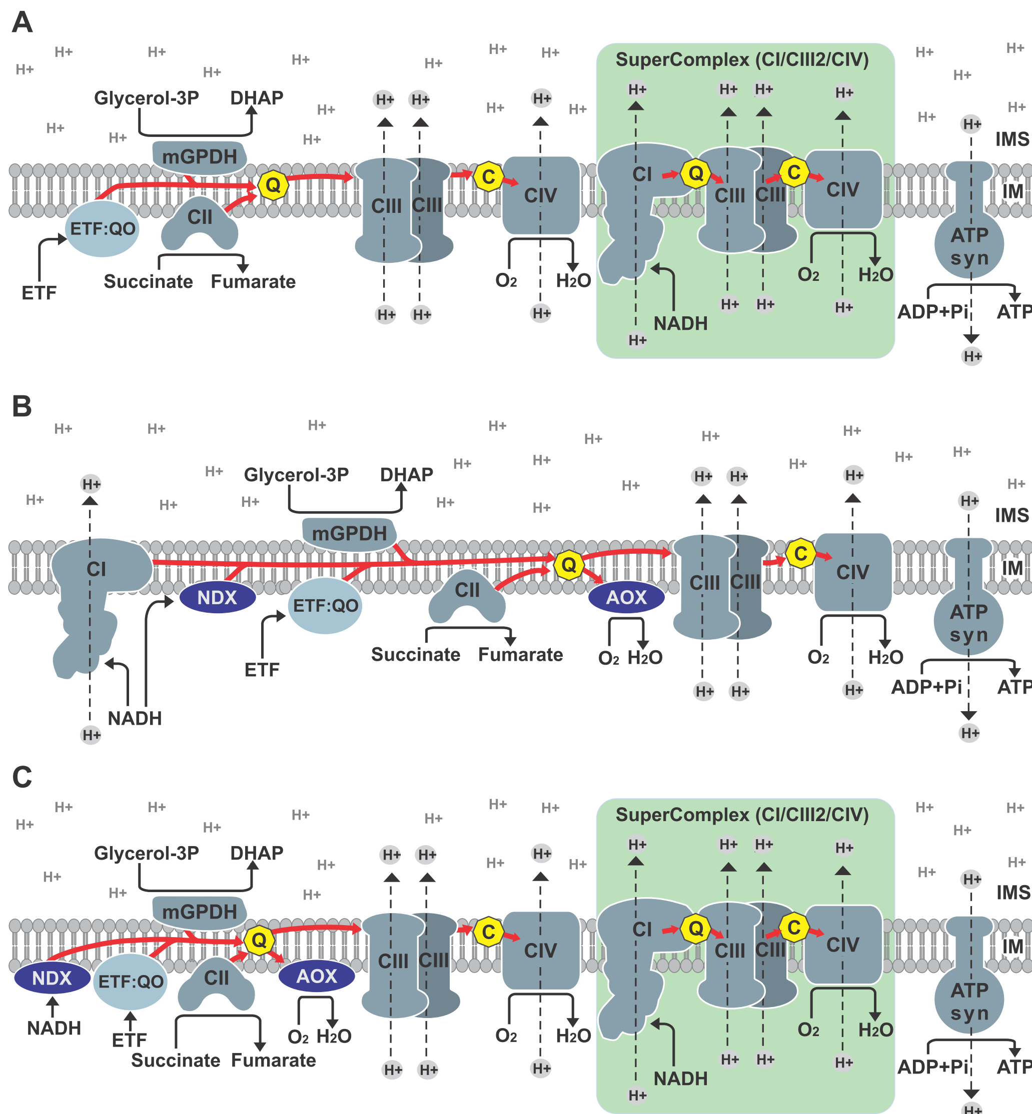

Fig. 6. Proposed distinct respiratory chain organizations in animals. A, The respiratory chain of mammals (and other vertebrates?) and insects, showing individually floating enzymes (*left side*) and supercomplexes (light green rectangle, *right side*). The latter is proposed to lower ROS production by facilitating electron transfer (red arrows) from NADH to O_2. Note the possible separate coenzyme Q (Q) and cytochrome *c* (C) pools created by supercomplex formation (see text for details). B, Putative arrangement of tunicate respiratory chain, showing the alternative enzymes NDX and AOX, and no SCs. The alternative enzymes may prevent excessive production of ROS via complexes I and III by lowering the over-reduced state of the OXPHOS components. C, Representation of how *Ciona* NDX and AOX might be arranged in the respiratory chain of the mouse and *D. melanogaster* when xenotopically expressed, excluded from supercomplexes. Note that the Q pool available for these alternative enzymes in this hypothetical scenario is different than postulated for tunicates or for when Complexes III/IV are dysfunctional and supercomplexes are disrupted. IM, mitochondrial inner membrane; IMS, mitochondrial intermembrane space; CI-IV, complexes I-IV; ATP syn, ATP synthase; H^+, protons; doted arrows, direction of proton pumping. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reflects their true absence in these organisms, it is also possible that their encoded polypeptides have been replaced by newly recruited proteins at the periphery of the OXPHOS complexes, which could putatively also allow SC formation with completely different arrangements. Although highly speculative at this point, this scenario would be likely to occur in organisms with fast evolving genomes, such as tunicates [96–99]. Tunicate AOX and NDX might even be capable of interacting with the OXPHOS complexes in their native systems through these completely new peripheral subunits, and hence have access to the CoQ pool partitioned by SCs. Based solely on the findings we describe here, tunicates do appear to have a less complex or significantly different canonical respiratory chain. Yet, they have retained AEs, whereas animals with more complex (or with classically arranged/structured) respiratory chains like vertebrates and insects have lost them. Considering that AEs and SCs might both prevent excessive ROS production, the former by helping reoxidize a larger pool of reduced CoQ [62] and the latter by diminishing the distances between the sites of CoQ reduction in CI and oxidation in CIII [65], we speculate AEs and SCs may serve comparable and somehow overlapping purposes in animals (Fig. 6). Whatever the reasons why vertebrates and insects lost AEs, a putative increase in SC formation after this event might have served as a compensatory mechanism to avoid excessive ROS without compromising mitochondrial ATP production. Conversely in tunicates, the loss of structural complexity (or the structural rearrangement) of the OXPHOS complexes, with possible absence of SCs, may have been a strong pressure for the retaining of alternative pathways to deal with the possibility of excess ROS formation. It is yet to be determined if our findings here are a universal phenomenon in animals or specific to the comparisons between tunicates, vertebrates and insects. In other words, do other AE-bearing animals also lack peripheral OXPHOS subunits important for SCs, and do other AE-nonbearing animals possess these subunits and/or are able to form SCs? The recent evidence for horizontal AOX gene transfer in some animal groups [18] is particularly interesting in this context, as at least in the case of the Sciaridae dipterans, having SC-forming genes is a characteristic inherited vertically from ancestral insects. It is noteworthy, though, that this speculative scenario of prevention of excessive ROS could also be promoted by the high abundance and the tight packing of OXPHOS complexes and electron carriers in the mitochondrial inner membrane, without necessarily high levels of SCs. This appears to be the case for the adult flight muscle mitochondria of *Drosophila*, in which SCs are present but are not the primary forms in which the OXPHOS complexes are found [100]. However, *Drosophila* SCs are physiologically relevant, as mild genetic perturbations of CI, III or IV can lead to increased levels of SCs in adults [58]. It is clear that most of the interpretations and conclusions throughout this manuscript are based upon analogies with the mammalian systems, so future research using different *Drosophila* tissues and other types of OXPHOS perturbations, and data from animal species other than vertebrates, insects and tunicates is welcome and may uncover valuable insights into cellular respiration adaptations and the regulation of ROS production.

Our findings also have implications for the biotechnological use of AEs in putative therapies. AEs constitutively and ubiquitously expressed in mammalian and insect models were originally envisioned to be functionally neutral, unless a large pool of reduced CoQ became available due to CIII and/or IV malfunction [25,60]. This was inferred especially based on classical data on plant AOX, which shows that this enzyme has a much higher K_M for reduced CoQ than CIII [101–103]. However, the engagement of *Ciona* AOX with non-SC dehydrogenases of the mammalian/insect respiratory chain changes the balance of reduced CoQ molecules available in the mitochondrial inner membrane, especially considering that a substantial number of CIII is committed in SCs, at least in humans [4,5]. Therefore, AOX has the potential to become functional in conditions that are more difficult to predict, possibly increasing the flux through and/or regulating specific metabolic pathways. Increased flux through CII-AOX, for example, would uncouple mitochondria, but also accelerate the TCA cycle, which in turn would provide more NADH for the coupled CI/III_2 or CI/III_2/IV SC electron transfer pathways. ATP production could thus be compensated by increased NADH oxidation. In the case of the *Drosophila* larvae, the mGPDH-AOX functional engagement, which also uncouples mitochondria, appears to downregulate mGPDH in the OXPHOS state (Fig. 5A, black bars in panels *i* and *ii*). This enzyme is naturally leaky ([104] and Fig. 5B, compare black bars in panels *v* and *vii*), shown to be thermogenic in other insects [105]. We speculate that mGPDH activity/protein level is regulated by its leakiness, so the increased uncoupling promoted by the functional interaction with AOX might lead to this downregulation of mGPDH, in an attempt of the cells/tissue to prevent excess uncoupling. mGPDH is also a component of the glycerophosphate shuttle, important to stream reductive power from glycolysis directly to the respiratory chain and to reoxidize NADH in the cytoplasm [104]. Downregulation of mGPDH would increase the ratio of its substrate glycerol-3-phosphate to the product dihydroxyacetone-phosphate, possibly forcing the larvae to make more use of another cytosolic NADH reoxidation pathway, such as the reaction catalyzed by lactate dehydrogenase (Ldh). In fact, the balanced action of the glycerophosphate shuttle and Ldh has been reported for *Drosophila* during larval growth [106], and we have evidence that *Ldh* transcripts are substantially elevated in AOX-expressing larvae [107].

The functional interactions of *Ciona* AOX with non-SC dehydrogenases of the mouse and the fly may explain the contrasting effects seen when AOX is expressed in these AE-nonbearing animals, ranging from beneficial to neutral to detrimental. To make the possible AOX therapies more realistic, we envision two — not necessarily exclusive — possibilities on which research may progress. First, a system for regulating at the transcriptional level its xenotopic expression in humans and in animal models must also be developed and tested; it would be ideal if the AE polypeptides were only produced when necessary, perhaps upon SC rupture and/or elevated mitochondrial ROS. This would reduce the chances of AOX engaging with non-SC dehydrogenases under normal physiological conditions, and would mimic more closely the natural regulation of the enzyme seen in plants [61,108] and even in *C. intestinalis* [28,63]. An AOX messenger RNA-based transfection system for human cells has already been developed [109] and could be used with the purpose of only adding AOX when (and where) AOX is needed. Second, there exists a magnificent possibility of the animal kingdom having a wide range of conventional respiratory chain and AE combinations, including SCs and no AEs (like in mammals and insects), no SCs and AEs (like we are speculating here for tunicates, based on the lack of several OXPHOS genes), and SCs plus both (or either) AEs (like in the putative cases of lateral AOX gene transfer in rotifers, some hexapods and plant-parasitic nematodes [18]). We speculate that animal AEs that have evolved and function naturally in a mitochondrial environment with conventional SCs are more likely to function more appropriately when xenotopically expressed in mammalian and insect models. AOX genes from different plant species have been expressed successfully in human cells in culture [110–112], but it is yet to be shown how they behave in a more complex system, such as whole animal models, if plant SCs are functionally equivalent to animal SCs, and most importantly if these plant species in particular also form SCs naturally. We find the structural diversity of the respiratory chain within metazoans more appealing to be explored for future successful expression and function of AEs in humans, although much has yet to be discovered and studied across the animal kingdom.

Our findings here only scratch the surface on the potential contribution animal AEs may have on their use in putative therapies and on how they impact the evolution, organization and function of the respiratory chain. Considerations regarding their potential thermogenic properties must also be made, especially in the context of the availability of oxidized/reduced CoQ molecules outside SCs in the mitochondrial inner membrane of mammals and insects. Although AOX appears not to alter thermal physiology in the endothermic mouse [22,26,113], in the ectothermic fruitfly, its expression has a significant impact on

development and viability under cold stress [28]. Glycerol-3-phosphate oxidation by mGPDH in the bumblebee *Bombus terrestris* has been shown to be important for pre-flight thermogenesis in cold temperatures, supporting CI-linked respiration and ATP production in this organism's flight muscles [105]. The mGPDH-AOX pathway shown here for *Drosophila* might provide a similar pre-heating condition for CI-based metabolism that would explain the cold stress resistance described above, but this has yet to be shown. We envision that showing if and how AEs actually also contribute to body heat production and thermal stress resistance in animals naturally bearing AEs is just a matter of time.

4. Materials and methods

4.1. Retrieval of OXPHOS subunit genes

The ID and names of the genes for all the subunits of the OXPHOS complexes of *H. sapiens* and *D. melanogaster* were obtained from the HUGO Gene Nomenclature Committee (HGNC [114]) and FlyBase [115], and confirmed manually through analyses of the scientific literature [3,53,116–119]. Their amino acid sequences were retrieved from the curated RefSeq [120] database for humans and *D. melanogaster*, and used in tBLASTn v 2.15 [121] searches against the *C. robusta* (former *C. intestinalis* type A), *C. intestinalis* (former *C. intestinalis* type B) and *C. savignyi* nucleotide sequence data available at NCBI non-redundant protein (nr-NCBI) database [120], Ensembl [122], and the curated ascidian database Aniseed [123]. It is important to note that the *C. intestinalis* genome assembly in the Ensembl and nr-NCBI databases refer to *C. robusta* (former *C. intestinalis* type A). The *C. intestinalis* (former *C. intestinalis* type B) genome assembly is currently listed as draft, so the OXPHOS gene sequences were manually assembled based on nucleotide and amino acid sequence similarities with the *C. robusta* and *C. savignyi* sequences. The predicted amino acid sequences retrieved were submitted to the TargetP, v 2.0 [124], and Deeploc2, v 2.0 [125], software to infer the probability of the polypeptide being targeted to mitochondria. The searches for the putatively missing OXPHOS subunit genes in other tunicate species were performed using BLASTp with the PSI-BLAST algorithm, v 2.15 [126], a threshold of 0.005 and three iterations using results only from the RefSeq database to generate the position-specific scoring matrix in the nr-NCBI. We also performed HMM-based homology searches using the HMMER web server, v 3.4 [127], with default parameters. When any tunicate sequence was retrieved, it was subsequently used in tBLASTn searches against the curated Aniseed database.

4.2. Structure analyses and modeling

The amino acid sequence alignments shown in Suppl. Figs. S1, S3, S5-S7 were performed using MUSCLE, v 5.0 [128] with default parameters, and manually adjusted for better visualization using ALTER, v 1.3.4 [129]. Homology models of the retrieved amino acid sequences of the *C. robusta* OXPHOS subunits were generated using the default parameters of I-TASSER, v 5.2 [46] and of the AlphaFold2-based platform ColabFold, v 1.5.5 [47,48]. These models were then compared to existing structures of the mammalian OXPHOS complexes and SCs (PDB IDs: 5Z62, 5GPN, 5XTH, 5J7Y and 5J4Z) using PyMOL, v 2.4.1 (https://www.schrodinger.com/pymol). The subunits important for SC formation and stability, and the interaction points between complexes were evaluated according to the literature [2,3,9], and used to discuss the relevance of the PMT subunits in *Ciona*.

4.3. Fly lines and quantitative PCR analyses

The fly line w^{1118} was used as an AOX-nonexpressing control. The line 3x*tubAOX*, which carries three copies of the *C. intestinalis* AOX coding sequence under control of the constitutive *Drosophila αTub84B* gene promoter in the w^{1118} background [33], was used as the experimental line. The flies were cultured at 25 °C, with 12-h light/dark cycle, and on standard diet, as described previously [23].

Four whole wandering third instar (L3) larvae (~120 h post-oviposition) were collected and fast frozen in liquid nitrogen. Total RNA was extracted by tissue homogenization in the presence of 300 μl TRI Reagent™ Solution (Invitrogen™), followed by addition of 120 μl DEPC water, vortexing for 15 s and incubation at room temperature for 15 min. The samples were centrifuged for 20 min at 12,000 ×*g*, and the supernatant transferred to a new tube. 300 μl ice-cold isopropanol was added, and the samples were centrifuged as previously. The RNA pellet was washed twice with 500 μl ice-cold 75 % ethanol, centrifuged at 12,000 ×*g* for 10 min, resuspended in 30 μl RNase-free water and stored at -80 °C. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) and 1 μg of total RNA were used for cDNA synthesis, according to the manufacturer's specifications. Quantitative real-time PCR experiments were performed on the CFX Opus Real-Time PCR System (Bio-Rad) using the PowerUp™ SYBR™ Green Master Mix for qPCR (Applied Biosystems™), according to the manufacturer's recommendation. Relative transcript quantification was calculated using the ΔΔCt method and the amplification data obtained using the cDNA as template and the primers: 5′TCCTCGGTGGGCGTTGGATT3′ and 5′CGACGGCCGCGTCATCTTCT3′ for *Gpo1*; 5′ATTTTCTTTGGCTTACTTAATCTCAC3′ and 5′CAATTTCTGGCGCTTTCA3′ for *AOX*; and 5′TCGACAACAGAGTGCGTCGC3′ and 5′CTTGAATCCGGTGGGCAGCA3′ for the reference gene *RpL32*. These experiments were replicated twice.

4.4. Mitochondrial oxygen consumption assays

Mitochondrial oxygen consumption rates were assessed in homogenates of wandering L3 larvae using the high-resolution respirometer O2k (Series D-G; Catalog Number 0000-02; Oroboros Instruments), according to [130]. Detailed information on reagents is described in Supp. Table S5. Twenty individuals were randomly collected using a thin paintbrush, rinsed in distilled water, and processed in an ice-cold hand glass homogenizer (Sartorius) containing 1 ml of homogenization buffer (250 mM sucrose, 5 mM Tris HCl, 2 mM EGTA, pH 7.4). The resulting homogenate was transferred into an ice-cold microtube, from which 100-200 μl were removed and added into the O2k respiration chambers containing 1850-1950 μl of respiration medium (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, 2 % BSA, pH 7.2), with constant stirring at 750 rpm and temperature of 25 °C. The remaining homogenate was stored at −20 °C for later protein quantification using the Bradford method.

To measure CI-driven oxygen consumption, 5 μl of 2 M pyruvate (final concentration: 5 mM) and 7.5 μl 0.4 M malate (1.5 mM) were added. For mGPDH-driven oxygen consumption, 80 μl of 0.65 M glycerol-3-phosphate (26 mM) and 1 μl 0.01 M rotenone (0.005 mM) were added. Next, we analyzed either of two mitochondrial respiration states commonly examined in mitochondrial physiology studies. The OXPHOS state was reached by addition of 4 μl 0.5 M ADP (1 mM), whereas the Leak state was induced using 1 μl 25 μM oligomycin (12.5 nM). For each CI- or mGPDH-driven measurements at both OXPHOS and Leak states, respiration sustained exclusively by AOX was verified by adding 2 μl 0.05 M antimycin A (0.05 mM), followed by 2 μl titrations of 0.1 M propyl gallate (0.1-0.2 mM); the inverse order of inhibitors was used to quantitate CIII/IV-sustained oxygen consumption in a parallel experiment. All respiration values were determined by subtracting the residual respiration following inhibition of CI, CIII and AOX, and were normalized by the total protein content in the larval homogenates. Statistical analyses were performed using the Jamovi software (https://www.jamovi.org), as described in the legend to Fig. 5.

4.5. Blue native-polyacrylamide gel electrophoresis analyses

Mitochondrial preparations were performed using 10 adult individuals (~2 days post-eclosion) or 100 wandering L3 larvae (~120 h

post-oviposition), according to [54,131,132], with adaptations. Briefly, the individuals were homogenized in 700 μl ice-cold isolation buffer (250 mM sucrose, 10 mM Tris HCl, 0.15 mM $MgCl_2$, pH 7.4 + 1X Thermo Scientific™ Halt™ Protease Inhibitor Cocktail), followed by two rounds of centrifugation at 4 °C, the first at 600 ×*g* for 5 min and the second at 9000 ×*g* for 10 min. The pellets were resuspended in 300 μl isolation buffer and protein quantification was performed following the Bradford method. Approximately 50 μg mitochondrial proteins were then recentrifuged at 9000 ×*g* for 10 min, and the pellets resuspended in 20 μl 1× NativePAGE™ Sample buffer containing 1.5 % digitonin and incubated on ice for 20 min. The samples were then centrifuged at 20,000 ×*g* for 20 min and 1.5 μl Comassie Blue G-250 Sample additive 5 % (Invitrogen™) were added to the supernatants. The processed samples were electrophoresed at 4-8 °C in 3-12 % NativePAGE™ precast Mini Protein gels, using NativePAGE™ Anode and Light Blue Cathode Buffers (Invitrogen™), at 150 V for 30 min, at which point the Light Blue Cathode Buffer was replaced by a dye-free Cathode Buffer and the run continued at 200 V for further 150 min. The gels were stained with Colloidal Blue Staining Kit (Invitrogen™) and imaged using the ChemiDoc MP Imaging System (BioRad). Data shown in Supp. Fig. S11B were obtained using mitochondrial extracts treated with OXPHOS inhibitors, as described in the figure legend, for 30 min on ice prior to solubilization with digitonin and BN-PAGE.

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CRediT authorship contribution statement

Murilo F. Othonicar: Writing – review & editing, Methodology, Investigation, Formal analysis. **Geovana S. Garcia:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Marcos T. Oliveira:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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