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# Respiratory Complexes III and IV Are Not Essential for the Assembly/Stability of Complex I in Fungi

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The functional relevance of respiratory supercomplexes in various eukaryotes including mammals, plants, and fungi is hitherto poorly elucidated. However, substantial evidence indicates as a major role the assembly and/or stabilization of mammalian complex I by supercomplex formation with complexes III and IV. Here, we demonstrate by using native electrophoresis that the long-lived *Podospora anserina* mutant *Cyc1-1*, respiring exclusively via the alternative oxidase (AOX), lacks an assembled complex III and possesses complex I partially assembled with complex IV into a supercomplex. This resembles the situation in complex-IV-deficient mutants displaying a corresponding phenotype but possessing I–III supercomplexes instead, suggesting that either complex III or complex IV is in a redundant manner necessary for assembly/stabilization of complex I as previously shown in mammals. To corroborate this notion, we constructed the double mutant *Cyc1-1,Cox5::ble*. Surprisingly, this mutant lacking both complexes III and IV is viable and essentially a phenocopy of mutant *Cyc1-1* including the reversal of the phenotype towards wild-type-like characteristics by the several-fold overexpression of the AOX in mutant *Cyc1-1,Cox5::ble (Gpd-Aox)*. Fungal specific features (not found in mammals) that must be responsible for assembly/stabilization of fungal complex I when complexes III and IV are absent, such as the presence of the AOX and complex I dimerization, are addressed and discussed. These intriguing results unequivocally prove that complexes III and IV are dispensable for assembly/stability of complex I in fungi contrary to the situation in mammals, thus highlighting the imperative to unravel the biogenesis of complex I as well as the true supramolecular organization of the respiratory chain and its functional significance in a variety of model eukaryotes. In summary, we present the first obligatorily aerobic eukaryote with an artificial, simultaneous lack of the respiratory complexes III and IV.



*Keywords:* aging; alternative oxidase; complex I biogenesis; mitochondria; respiratory supercomplexes

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## Introduction

The mitochondrial oxidative phosphorylation (OXPHOS) system is the main generator of cellular ATP in most eukaryotes, whose major components are the four respiratory chain complexes and the $F_OF_1$-ATP synthase located in the inner mitochondrial membrane.[1,2] Complexes I (NADH:ubiquinone oxidoreductase), III (ubiquinol:cytochrome *c* oxidoreductase), and IV (cytochrome *c* oxidase, COX) transduce the energy of nutritional compounds into an electrochemical proton gradient across the inner membrane, used by the $F_OF_1$-ATP synthase (complex

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Abbreviations used: AOX, alternative oxidase; OXPHOS, oxidative phosphorylation; BN, blue-native; NADH-DH, NADH dehydrogenase; CN, colorless-native; COX, cytochrome *c* oxidase; DBQ, decylubiquinone.

V) to generate ATP. Strong evidence has been accumulating that in most eukaryotes,[3–11] including filamentous ascomycetes,[12,13] complexes I, III, and IV are organized as stoichiometric supercomplexes and the ATP synthase is organized as homodimers/homooligomers (reviewed in Refs. [14–18]). Of particular importance is the recent determination of single-particle structures of respiratory supercomplexes.[19–22] The functional significance of respiratory supercomplexes is still poorly understood, but there is substantial evidence that one of their major roles is the assembly and/or stabilization of complex I at least in mammals.[5,23–26]

Unlike mammals, many fungi, plants, algae, and protists possess alternative respiratory enzymes branching the standard respiratory chain without energy-conserving proton pumping, particularly the alternative NADH:ubiquinone oxidoreductases[27–31] and the so-called alternative oxidase (AOX).[29,32] The latter bypasses complexes III and IV, is insensitive against cyanide and other COX inhibitors, and was even confirmed to occur in some lower animals.[33]

Filamentous fungi such as the pezizomycete (formerly euascomycete) *Neurospora crassa* are invaluable model organisms necessary to explore essential molecular features of eukaryotes,[34] for example, in mitochondria research such as investigation of the protein import machinery (e.g., Refs. [35,36]) and the biogenesis of complex I.[30,31,37] In contrast to most fungi, capable of infinite vegetative growth, *Podospora anserina*, a close relative of *N. crassa*, is prone to a senescence process. *P. anserina* has been used as a model eukaryote to investigate molecular aspects of aging for decades, more so as a mitochondrial etiology, particularly the age-dependent systematic reorganization of mtDNA in all wild-type isolates is well established.[38–40] Of particular interest is the apparently causative link of respiration and longevity: The exclusive use of the AOX respiration due to the specific impairment of either complex IV,[41–43] for example, mutant *Cox5::ble*[42] lacking a gene for an essential COX subunit, or complex III (mutant *Cyc1-1*[44]) with a loss-of-function mutation in the gene encoding cytochrome $c_1$ of complex III leads to mtDNA stabilization and virtual immortality. However, the observation that the constitutive several-fold overexpression of the AOX in those mutants, *Cox5::ble (Gpd-Aox)*[45] and *Cyc1-1(Gpd-Aox)*,[44] restores senescence and other wild-type characteristics to a large extent is puzzling.

Here, we show that long-lived *P. anserina* mutants lacking either complex III or complex IV have I–IV or I–III supercomplexes, respectively, suggesting that either complex is involved in a redundant way in assembly/stability of complex I as previously shown in mammals. However, by constructing and analyzing the double mutant *Cyc1-1,Cox5::ble*, with it being a phenocopy of the single mutants and devoid of complexes III and IV, we prove that both complexes are not essential for assembly/stability of fungal complex I in contrast to the situation in mammals.

## Results and Discussion

### The long-lived complex-III-deficient mutant *Cyc1-1* has a I–IV supercomplex

To gain insight into the molecular basis of lifespan extension in cytochrome-deficient mutants, we analyzed the steady-state OXPHOS system of *Cox5::ble*, *Cyc1-1*, the rescue mutants *Cox5::ble(Gpd-Aox)* and *Cyc1-1(Gpd-Aox)*, and juvenile wild-type mitochondria as a control. This was done by blue-native (BN) PAGE of mitochondria solubilized with the particularly gentle detergent digitonin able to preserve OXPHOS supercomplexes (e.g., Refs. [3–22,24–26,46,47]). For direct comparison of the respiratory chain in these strains, the BN gels were probed for in-gel activity of NADH dehydrogenase (NADH-DH; complex I) and COX (complex IV) (Fig. 1a). Second-dimension SDS gels gave the characteristic subunit patterns of OXPHOS complexes and supercomplexes (Fig. 1b and c). In line with previous results from *Podospora*[12] and *Neurospora*[13] wild-type mitochondria, large amounts of supercomplexes comprising complexes I, III, and IV ($I_1IV_1$, $I_1III_2IV_1$, and $I_xIII_yIV_z$) as well as the smaller ones ($III_2IV_1$ and $III_2IV_2$) along with ATP synthase monomers and dimers ($V_1$ and $V_2$) were found in our wild-type culture (Fig. 1a and b). As expected, the complex-IV-deficient mutants *Cox5::ble* and *Cox5::ble(Gpd-Aox)* displayed no bands with COX activity but a pattern of high-molecular-weight species with NADH-DH activity (Fig. 1a). Second-dimension SDS-PAGE (not shown) confirmed those bands to be monomeric and dimeric complex I ($I_1$ and $I_2$) as well as the I–III supercomplexes $I_1III_2$ and $I_2III_2$, previously found in very similar amounts in the mutants ex1 and grisea[12] likewise lacking complex IV. Importantly, since the mutants *Cox5::ble*, ex1, and grisea carry different mutations either in nuclear or in mitochondrial genes, these results suggest that any specific loss of complex IV leads to a characteristic AOX-dependent respiratory chain comprising monomeric and dimeric complex I as well as I–III supercomplexes.[12] In contrast, in the mutants *Cyc1-1* and *Cyc1-1(Gpd-Aox)* devoid of complex III activity,[44] the in-gel activity stainings immediately verified the presence of complex IV and monomeric complex I as the predominant complex I species (such as in the COX-deficient mutants) as well as a distinct band with both NADH-DH and COX activity (Fig. 1a). 2D BN/SDS-PAGE demonstrated that this high-molecular-mass species (~1250 kDa) is a supercomplex of each a monomer of complexes I and IV ($I_1IV_1$) (Fig. 1c), also found in the wild type (Fig. 1b), *N. crassa*,[13] and bovine heart[3,6] by the same approach. Significantly, this direct complex I–complex IV interaction was corroborated in 2D and 3D single-particle structures of the bovine heart supercomplex $I_1III_2IV_1$.[21,22] While complex I dimers could not be detected after digitonin solubilization of *Cyc1-1* (Fig. 1a and c) and *Cyc1-1(Gpd-Aox)* mitochondria (Fig. 1a), very