Response to Reviewers

Reviewer #1:

„1.It isn't clear what the major findings are in support of a predominant association of NDUFA4 with complex I under `normal' conditions, and a switch to associating with CytOX upon stress. This has to be much better elaborated."

I included in the revised manuscript on page 5: NDUFA4 was identified as a nuclear-encoded subunit of complex I [78-80]. However, together with two other subunits its gene had significantly increased amino acid substitution rates during primate radiation, suggesting that they have been subjected to adaptive selection [81]. Later NDUFA4 was no longer considered a subunit of complex I [82]." and later „These results strongly suggest that stress-dependent increase of cytosolic calcium leads to a rise of $\Delta\Psi_m$ and ROS formation at lower efficiency due to loss of the allosteric ATP-inhibition of CytOx [47]."

The binding of NDUFA4 to monomeric CytOx is demonstrated by the cryo-EM structure of the NDUFA4-CytOx complex. Monomerization occurs apparently by Ca^{2+}-dependent dephosphorylation of dimeric CytOx [1].

„2. A Ca2+-dependent phosphatase in the mitochondrial IMS seems to reciprocally regulate the affinity of both complexes for NDUFA4. What is the identity of the phosphatase (or any evidence that it is a phosphatase)?"

The identity of the phosphatase was concluded from switching off the „allosteric ATP-inhibition" of isolated CytOx from bovine heart by the Mn2+-activated protein phosphatase PP1, which was reversed by cAMP-dependent PKA [76], and of Tween-20 solubilized bovine liver mitochondria by addition of $CaCl_2$ [77]. An IMS-located phosphatase has not yet been identified.

„3. Is there any good evidence that CytOX complex preferentially associates with NDUFA4 in its monomeric form? For that matter is there any evidence that NDUFA4 binding will stabilize monomeric CytOX? The author cited Pitceathly et al. (2013) as providing evidence that NDUFA4 acts as a stabilizer of monomeric CytOx. However, in the paper the NDUFA4 mutation resulted in undetectable steady-state NDUFA4 protein levels and loss of CytOX function. If so, does it mean that the CytOX is not functional in its dimeric form?"

The binding of NDUFA4 to the 13-subunit CytOx complex was demonstrated by its cryo-EM structure [87]. Pitcealthly showed cleavage of the NDUFA4-CytOx complex already at 0.08% dodecylmaltoside [84]. In Western blots of muscle tissue from Leigh syndrome patients with defective CytOx activity and identified mutations in the *NDUFA4* gene the CytOx complex was still there but NDUFA4 was completely absent. We do not know the function of NDUFA4 and whether it stabilizes the monomeric CytOx. Monomeric and dimeric CytOx are active [1], also shown with the isolated enzyme [76]. I changed the text on page 6 as follows:

„The role of NDUFA4 as 14th subunit of COX was suggested by [84] based on mutations in the *NDUFA4* gene accompanied by defective COX activity in patients with Leigh syndrome. In muscle tissue from patients the NDUFA4 protein was absent while the CytOx complex was still there but without activity. Since the literature is full of papers measuring CytOx activity with the isolated 13-subunit enzyme (without NDUFA4), the physiological function of NDUFA4 remains unknown. We suggest to rename it to „mitochondrial respiratory chain associated factor 1". "

„4. A glaring omission in the manuscript is a detail discussion of how might future work support or refute the author's proposed hypothesis. Any good hypothesis should be falsifiable

experimentally. The author should therefore outline the experiments that should be done in the immediate future.“

A dynamic change of protein-protein interactions is part of cellular life. This is strongly influenced by protein phosphorylations which change the affinities between proteins. The proposed hypothesis involves reversible phosphorylation of CytOx subunit I on the intermembrane side, and of complex I at an unknown site. The hypothesis could be further verified by BN-PAGE of mitochondria which have been either treated with cAMP + PKA or with 10 micromolar calcium as described in [1]. The gels should be immunolabeled with antibodies against complex I, CytOx, and NDUFA4. But the methodological details are not subject of this review article.

Reviewer #2:

„Minor Issues: Based on the title, one would expect to see additional information on the health and disease implications of the cytochrome c oxidase, but the manuscript focuses mainly on the physiological aspects.“

I have included on page 2: „Health and optimal life are frequently hurt by the consequences of psychosocial stress.

The consequences appear in cells as „oxidative stress“ caused by the over-production of reactive oxygen species (ROS, mainly $O_2^{\cdot -}$ and H_2O_2) in mitochondria. While low amounts of ROS have in cells signalling functions [6,7], high amounts produced in mitochondria are generally assumed to participate in aging [8-10] and in the generation of numerous diseases including cancer, hypertension, atherosclerosis, ischemia/reperfusion injury, neurodegenerative diseases like Alzheimer's and Parkinson's disease, rheumatoid arthritis, diabetes mellitus, and mitochondrial diseases [11-14].”

And on page 4: “The low ROS production in mitochondria of living cells under resting conditions [18] is thus explained by the allosteric ATP-inhibition of CytOx which maintains low $\Delta\Psi_m$ values [46]. Therefore this mechanisms contributes to the health and optimal life of higher organisms.”

„The overall novelty is unclear, since, besides a hypothesis, their elements of novelty are not clearly stated. Although a scheme regarding the Hypothesis on the variable binding of NDUFA4 to complex I or CytOx is provided (Fig. 1), additional graphical presentations of the content of the manuscript will make the information more comprehensible, easier to follow and finally insease the value of the material.“

The main basis of this hypothesis is the „allosteric ATP-inhibition of CytOx“ which was published and discussed in more than 20 publications (often ignored). Additional graphical presentations on the physiological meaning of the „allosteric ATP-inhibition of CytOx“ are shown in the papers: [46-48].

„Please correct the few typos that can be found across the manuscript, as: -“work load” - „Bioenergetics4“ - “Villani et bal., 1998”“.

The typos have been corrected.

Reviewer #3:

„1. The title is irrelevant to the content of the paper. The authors have not described the health and optimal health of any animal or organ or cells. They only describe a potential mechanism to control the activities of a component in electron transport chain by NDUFA4 and its relationship to ATP regulation.“

In the revised manuscript I included under the chapter “Reactive oxygen species (ROS) in mitochondria”: “Health and optimal life are frequently hurt by the consequences of psychosocial stress. The consequences appear in cells as „oxidative stress“ caused by the over-production of reactive oxygen species (ROS, mainly $O_2^{\cdot -}$ and H_2O_2) in mitochondria. While low amounts of ROS have in cells signalling functions [6,7], high amounts produced in mitochondria are generally assumed to participate in aging [8-10] and in the generation of numerous diseases including cancer, hypertension, atherosclerosis, ischemia/reperfusion injury, neurodegenerative diseases like Alzheimer's and Parkinson's disease, rheumatoid arthritis, diabetes mellitus, and mitochondrial diseases [11-14].”

In isolated mitochondria at state 4 (low ADP, high ATP, no ATP consumption = resting state) high amounts of total oxygen consumed by isolated mitochondria (2%) was shown to be converted into H_2O_2 [Chance et al., (1979) Physiol. Rev. 59(3), 527-605]. I describe a mechanism, the „allosteric ATP-inhibition of CytOx“, which prevents ROS production under „resting conditions“. In addition we explain mitochondrial „hyperpolarization“ as a consequence of stress factors and associated with increased ROS production by switching off the „allosteric ATP-inhibition of CytOx“.

„2. The first sentence of the abstract is not accurate. There are two ways to generate ATP in a cell, substrate phosphorylation and oxidative phosphorylation. The later only occurs in cells with mitochondria.“

The abstract was corrected: „The generation of cellular energy in form of ATP occurs mainly in mitochondria by oxidative phosphorylation.“ In the following the abstract was completely rewritten.

„3. The abstract does not include sufficient and useful information to support the hypothesis and conclusion.“

The abstract was completely rewritten.

„4. If you believe that ATP functions as an allosteric inhibitor of CytOx, please provide the concentration by which 50% inhibition occurs. In addition, please also indicate how this concentration relates to physiological concentrations of ATP/ADP in the mitochondrion. Please summarize data and include them to support your claim.“

On page 3 was added: „The ATP/ADP ratio in the mitochondrial matrix for half-maximal inhibition of CytOx activity at ATP/ADP = 28 [45] corresponds to the high cytosolic ATP/ADP ratio of 100-1000 determined by ^{31}P-NMR measurements in rat heart [50]. Due to $\Delta\Psi_m$ the ATP/ADP-ratio in the mitochondrial matrix will be lower (ATP/ADP = 4 - 40, see [47]).“

„5. There are many types of cells that serve variety of physiological functions. Please also elaborate the cell types that your hypothesis may apply to.“

On page 4 was now included: „The allosteric ATP-inhibition of CytOx is active in most cell types which express subunit IV-1. The isoform subunit IV-2 was found to be expressed in human cell lines under hypoxia [73]. Also in isolated astrocytes and cerebellar granule cells subunit IV-2 is expressed under hypoxic conditions accompanied by an abolition of the allosteric inhibition of COX by ATP [74].“

„6. Please do not quote a lot of sentences from other studies as those are points in other papers. They only work in the context of the paper. Please describe those points in your own language.“

The direct quotations have been removed in the revised manuscript.

Reviewer #4:

„1. It isn't clear what the major findings are in support of a predominant association of NDUFA4 with complex I under `normal' conditions, and a switch to associating with CytOX upon stress. This has to be much better elaborated. Are there any direct evidence for a preferential association by NDUFA4 with either complexes that is cytosolic Ca2+-dependent, or dependent on energy demand?“

The binding of NDUFA4 to complex I was discovered during purification and characterization of complex I mainly by the group of Walker/Cambridge [78-80]. But in the paper of Mishmar et al., [81] they found for NDUFA4 increased amino acid substitution rates and suggested an adaptive selection during primate radiation. Later NDUFA4 was no longer considered a subunit of complex I [82]. The binding of NDUFA4 to CytOx was concluded from its cryo-EM structure published by Zong et al., [87] and from the data of Balsa et al. [83]. The problem of binding NDUFA4 to complex I or CytOx is the unknown phosphorylation state of these complexes which influences the binding. We only know that phosphorylation of CytOx subunit I at the cytosolic side by a cAMP-dependent PKA leads to switching on the „allosteric ATP-inhibition and thus to its dimerization without NDUFA4 [76].

„2. A Ca2+-dependent phosphatase in the mitochondrial IMS seems to reciprocally regulate the affinity of both complexes for NDUFA4. What is the identity of the phosphatase (or any evidence that it is a phosphatase)?“

The evidence that a phosphatase switches off the allosteric ATP-inhibition of CytOx was demonstrated by use of protein phosphatase 1 (PP1) [76], and we correlated it with monomerization of CytOx [1]. Since the addition of only calcium to intact isolated heart mitochondria switched off the allosteric ATP-inhibition, a Ca^{2+}-activated protein phosphatase in the intermembrane space was concluded to be responsible, since a phosphorylation of CytOx subunit I at the cytosolic side was identified for switching on the allosteric ATP-inhibition [76]. The identity of the intermembrane space protein phosphatase, however, remains to be investigated.

„3. Is there any good evidence that CytOX complex preferentially associates with NDUFA4 in its monomeric form? For that matter is there any evidence that NDUFA4 binding will stabilize monomeric CytOX? The author cited Pitceathly et al. (2013) as providing evidence that NDUFA4 acts as a stabilizer of monomeric CytOx. However, in the paper the NDUFA4 mutation resulted in undetectable steady-state NDUFA4 protein levels and loss of CytOX function. If so, does it mean that the CytOX is not functional in its dimeric form?“

In the paper of Pitceathly et al. [84] the NDUFA4 mutation resulted in undetectable steady-state NDUFA4 protein levels but the CytOx protein was still there (unchanged), however, without activity. So the function of NDUFA4 on CytOx activity is completely unknown. The isolated CytOx shows activity, independent of its structure as monomer, dimer or polymer, however, with different activities [Rosevear et al., Biochemistry (1980) 19(17): 4108-4115]. The dimeric CytOx is active with "allosteric ATP-inhibition" in intact isolated rat heart mitochondria [1].

„4. A glaring omission in the manuscript is a detail discussion of how might future work support or refute the author's proposed hypothesis. Any good hypothesis should be falsifiable experimentally. The author should therefore outline the experiments that should be done in the immediate future.“

The answer to this argument is already given in the 4th point of **Reviewer #1**:
„A dynamic change of protein-protein interactions is part of cellular life. This is strongly influenced by protein phosphorylations which change the affinities between proteins. The proposed hypothesis involves reversible phosphorylation of CytOx subunit I on the intermembrane side, and of complex I at an unknown site. The hypothesis could be further verified by BN-PAGE of mitochondria which have been either treated with cAMP + PKA or with 10 micromolar calcium as described in [1]. The gels should be immunolabeled with antibodies against complex I, CytOx, and NDUFA4. But the methodological details are not subject of this review article.“