I thank Prof. Dux for the comments.

My responses inline (>Response).

Question 1. The sophisticated transport model developed and used by the author does not include the impact of Calcium ion trafficking, meanwhile their uptake and impact, both on the transmembrane potential gradient, and as a competitor for the inorganic Phoshate, available at the matrix side is documented and discussed in basic pathobiochemistry textbook since long. What was the reason not to include the traffic of the divalent kation Calcium into the rather comprehensive calculation model? How the model would behave under conditions when Calcium acculumation in the matrix space occurs?

>Response: The core of the transport model relies on the differential affinity of ligands such as ions on adenine nucleotides, in order to reliably estimate ATP appearing (or consumed) in the medium using standard binding equations. As such, we considered ATP and ADP as the main nucleotide participants, and $[Mg^{2+}]$ and $[H^{+}]$ as main ionic ligands. However, $[Ca^{2+}]$ is an additional ionic ligand for both ATP and ADP. Furthermore, Ca^{2+} complexates with inorganic phosphate. If we were to include $[Ca^{2+}]$ as an additional ATP- and ADP ligand, the following two issues would arise: i) the complexity of the system would increase enormously, and ii) the confidence of the calculation of [ATP] from the binding equations would diminish considerably. Point (ii) can be better appreciated by looking at figure 1.2 (page 44), panels C and D. The less the $[Mg^{2+}]/[ADP]$ or $[Mg^{2+}]/[ATP]$, the greater the signal-to-noise ratio of the calculated $[ATP]_{free}$ (i.e. compare trace e' with trace h' in panel D). The presence of Ca^{2+} in the media would practically exert the same effect, by minimizing the amount of free [ATP] and free [ADP] available for complexating with $[Mg^{2+}]$. As it can be envisaged in trace h' of panel D in figure 1.2, fitting a linear regression for calculating the rate of ATP appearing in the medium would exhibit a low coefficient of determination. Because of this, we recommend to fellow investigators who wish to use the method and the model to not use buffers that contain additional ligands such as creatine or creatine phosphate and ensure that chemicals are not contaminated with Ca^{2+}, Ba^{2+}, Mn^{2+}, Sr^{2+} or other metals that could complexate with ATP and/or ADP, https://semmelweis.hu/biokemia/en/research/christos-lab/antactivity/ (tab 5.), and this was also the reason not to include Ca^{2+} in the model. Regarding the specific question as of how would the model behave under conditions of matrix calcium accumulation: although the mitochondrial matrix exhibits enormous capacity for calcium sequestration, it does so by precipitating it with nucleotides, ions (especially phosphate), proteins and probably other molecules (Kristian et al. 2007); by doing so, the *free* $[Ca^{2+}]$ in the matrix remains almost unaffected even after accumulating a very large molar fraction of calcium, thus it would not disturb the parameters of the model to a significant degree. However, apart from causing mitochondrial depolarization, Ca^{2+} has an effect on many other entities within mitochondria such as the matrix dehydrogenases and potentially the Fo-F1 ATP synthase; thus indirect effects are expected but cannot be quantified, because calculation of extramitochondrial [ATP] (required for the model) in the presence of calcium cannot be made for the reasons elaborated above.

Question 2. Brown adipose tissue mitochondria are unique in their controlled uncoupling phenomena through Thermogenin channel proteins. Is there any evidence about the mitochondrial Adenine Nucleotide Transporter function in these tissues? Is there any relevance of ANT systems and mitochondrial substarete level Phosphorylation in the thermoregulation of the body? Does it have any possible involvement in body weight control with UCP protein expressions in human white adipose tissue?

>Response: Currently, this a hotly debated topic of research: regarding the adenine nucleotide transporter and uncoupling, an array of functional assays by a multitude of laboratories led to the conclusion that up to 70% of proton leak during state 2 or 4 (when the ANT does not transport nucleotides) is attributed to an intrinsic property of the ANT allowing protons through it and thus across the inner mitochondrial membrane. Proton leak by UCP1 and ANT was collectively referred to as "inducible leak", activated by fatty acids, superoxide or lipid peroxidation products (Jastroch et al. 2010).

However, in 2019, the concept of proton leak through the ANT was challenged by a crystallographic study showing no possibility for proton passage through this entity (Ruprecht et al. 2019). This view was subsequently refuted in 2022 by an electrophysiological study showing that ANT exhibits not only proton leak, but the authors also concluded that uncouplers facilitate proton leak through the ANT and UCP1 (Bertholet et al. 2022). In an even steeper departure from 'commonly accepted' bioenergetic concepts, it was also reported that UCP1 is not the means for conferring thermogenic properties to brown (and beige) adipose tissue, but this is due to futile cycling of creatine metabolism (Kazak et al. 2015). In turn, this has been critically cross-examined by Nicholls and Brand -pioneers in UCP1 and thermogenesis research- who discredited the results (Nicholls and Brand 2023) reported by the Spiegelman group that proposed the UCP1-independent, creatine-driven thermogenesis. In my opinion, without reaching first a consensus whether the ANT contributes to proton leak or not and whether UCP1 contributes to thermogenesis by intrinsic uncoupling or not, no concrete conclusions maybe drawn. Nevertheless, acknowledging that brown adipose tissue mitochondria are largely uncoupled (by whatever mechanism, UCP1 and/or futile creatine metabolism) it is expected that the ANT would operate exclusively in reverse, i.e. bringing ATP into the matrix; mitochondrial substrate-level phosphorylation would still be able to generate ATP within the matrix, but because the extent of membrane depolarization is expected to be almost complete, the reversal potential of the ANT should remain exceeded at all times, see figure 6.1, page 78; under these conditions, these mitochondria would exhibit ATPin/ADPin - $\Delta\Psi$m pair values that would place them in the "C" (grey) space, always bringing ATP into their matrix. Regarding the potential involvement of mitochondrial substrate-level phosphorylation in thermoregulation in brown adipose tissue and/or body weight control in connection to UCP1 expression in white adipose tissue: it has been reported that infusion of glutamine to healthy human subjects led to an increase in resting core temperature attributed to thermogenesis (Nakajima et al. 2004); although it is not known if that was due to downstream catabolism of glutamine or a potential effect of some glutamine-sensitive signal transduction mechanism, it is well known that glutamine is catabolized as follows: glutamine -> glutamate -> α-ketoglutarate -> succinyl-CoA -> succinate, thus passing through mitochondrial substrate-level phosphorylation. On the other hand, in preadipocytes (3T3-L1 cells) it has been reported that exposure to cold (which is known to induce UCP1 expression) the expression of a transporter carrying α-ketoglutarate across the inner mitochondrial membrane (oxodicarboxylate carrier) is downregulated (Niimi et al. 2009). α-ketoglutarate is catabolized through the above metabolite pathway (...-> α-ketoglutarate -> succinyl-CoA -> succinate) passing through mitochondrial substrate-level phosphorylation. In conclusion, data available regarding the potential connection between mitochondrial substrate-level phosphorylation, ANT, thermoregulation and UCP1 expression are rather scarce and circumstantial.

Question 3. How the major differences in mitochondrial enzyme/isoenzyme expression profiles among different brain cell types would fit to the endosymbiotic model of the mitochondrial origin? Which signalling pathways could be involved in the cell type specific differentiation of mitochondrial enzyme profiles and transporter systems?

>Response: Regarding the brain cell type specific mitochondrial enzyme/isoenzyme connection to the endosymbiotic model of the mitochondrial origin, this can be addressed by dwelling on the nucleus-based control of the mitochondrial proteome: Beforehand, I acknowledge that the question is for "brain-specific" cell types, however, the mechanisms that I will elaborate upon could apply for all cell types, although not all mechanisms unconditionally operate in all cells at the same time. Human mitochondria harbor ~1136 types of proteins, enlisted in the most recent version (3.0) of MitoCarta (Rath et al. 2021). Among them, only 13 proteins are encoded by the mitochondrial DNA, and the remaining 1123 from the nuclear genome. Apparently, the transition of allocating mitochondrially-localized protein encoding from mitochondria themselves (starting from the beginning of endosymbiosis 1.45 billion years ago) to the nucleus (to this day) occurred over the course of millions of years. As this time scale is the same for organism evolution, it is safe to assume that endosymbiotic mitochondria evolved together with the cells comprising the organism (Gray et al. 1999); as the organism evolved, exhibiting more and more specialized tissues and functions, harbored mitochondria evolved accordingly. Thus, it is easy to envisage

that mitochondria engulfed by specialized cell types assumed specialized roles and their proteomes were adjusted accordingly. For example, as we have recently uploaded in a pre-print server (the manuscript is under review) in the adult human neocortex and hippocampal formation there is such a stark cell-specific expression of key mitochondrial enzymes that precludes the possibility of OXPHOS in astrocytes (Dobolyi et al. 2022). We propose that the reason behind this is to support the Astrocyte-To-Neuron Lactate Shuttle (ANLS), a model dictating that in response to glutamate-mediated neuronal activity, astrocytes enhance their glycolytic flux forming lactate, which is shuttled to neurons through monocarboxylate transporters (Pellerin and Magistretti 1994). Without OXPHOS, human brain astrocytes are bound to produce lactate to avoid interruption of glycolysis. These findings support the assumptions elaborated above, i.e. astrocytic mitochondria abandoned the expression of OXPHOS enzymes for the sake of forming lactate which supports neuronal metabolism during firing; the evolution of neurons and astrocytes as distinct cell types set stage for the differential expression of proteins in their harboring mitochondria. Now, the question arises and as also Prof. Dux eluded to, which could be the signaling pathways responsible for cell type specific differentiation of mitochondrial proteomes? Mindful that differences in mitochondrial proteomes among cell types are under the control of the respective nuclei, the means of nucleus-mitochondria interaction must be examined. The nature of these interactions may be (i) physical and/or (ii) functional. Physical interactions between mitochondria and intracellular organelles are substantiated by "mitochondria- associated membranes" (MAM). Although stable MAM structures between mitochondria and nucleus have not been explicitly described, since the outer nuclear membrane is contiguous with the endoplasmic reticulum (ER) and the ER is in contact with mitochondria through well-established 'contact sites' mediated by MAMs, one may safely assume that the outer membranes of nuclei and mitochondria are also in contact. The physical interactions between nuclei and mitochondria may serve 'retrograde signaling', a means by which mitochondrial metabolites exert some effect on specific proteins (see below) traversing through MAMs, eventually communicating signals to the nucleus, eliciting a transcriptional response. The functional interactions between nuclei and mitochondria are much more complex and versatile: these interactions can be subdivided to those under (a) direct nuclear control (such as those affording mitochondrial targeting sequences, essentially dictating if the protein will end up in mitochondria or not, as well as transcription factors regulating the expression of entire mitochondrial pathways such as electron transport chain, steroidogenesis, mitophagy, etc) and (b) retrograde signaling involving diffusible messengers that do not require MAMs. Nuclear signals influenced by mitochondria could be a transcription factor (such as TFAM, CREB or NF-κB) or an enzyme (such as SOD2), in turn, mitochondrial signals that trigger a nuclear response may be affected by several different metabolites or redox-active metabolite pairs, such as succinate, acetyl-CoA $NADH/NAD^+$ and AMP/ATP. The exact mechanism responsible for dictating mitochondrial protein composition as a function of cell type ultimately depends on one or more among the hundreds of different mitochondrial-nucleus interaction pathways elaborated above, summarized in (Walker and Moraes 2022). Nevertheless, to address our specific findings, namely that astrocytes of the adult human neocortex and hippocampal formation lack mitochondrial enzymes for performing OXPHOS may be due to an inherent scarcity of Nrf2 (a transcription factor involved in functional, nuclear-mitochondrial interactions) in astrocytes: indeed, it has been reported that in human CA1 hippocampal region, Nrf2 stained the cytoplasm of GFAP-positive cells (i.e. astrocytes) only weakly, compared to the neurons (Ramsey et al. 2007); Nrf2 is well known to induce cytochrome oxidase subunit IV upregulation (Scarpulla 2006). Thus, the absence of OXPHOS in human astrocytes may be partly due to the inherent scarcity in Nrf2, the transcription factor for conveying the 'request' of mitochondria from the nucleus to receive complex IV protein.

Question 4. Heteroplasmic phenomena may alter the metabolic features in the frequently dividing tumour tissues. Does it have any positive or negative impact on the tumor progression process?

>Response: The phenomenon of mitochondrial heteroplasmy may be due to differences in the number of variants and mutations in mtDNA among cancer cells and/or within cancer cells. With the advent of interrogating mtDNA sequences in a single-cell manner, the current consensus leans towards the conclusion that heteroplasmy is due to inter-cellular rather than intra-cellular differences (Almeida et al.

2022). However, the question remains whether mtDNA mutations exert any discernible effect in the cancer cell or tissue (Ju et al. 2014), and if yes, whether it is causal or consequential regarding tumor progression. To this end, an enormous amount of data have been produced, mostly from the 'Pan-Cancer Analysis of Whole Genomes' (PCAWG) consortium (Yuan et al. 2020). However, from the work of PCAWG, the only definite answer that can be formulated regarding the question "does an increase in mtDNA heteroplasmy exert a positive or negative impact on tumor progression?" is that some somatic nuclear transfers of mtDNA disrupted therapeutic target genes, i.e. they may exert a positive effect in untreated tumor progression. A hefty amount of additional uncertainty and confusion regarding the role of mtDNA heteroplasmy and cancer, stems not from the concept that defects (due to mtDNA mutations) may lead to impaired OXPHOS, but whether OXPHOS defects exert any positive or negative effect on tumor progression. To this end, literature seems to be deeply divided; there are those considering that OXPHOS is intact and necessary for cancer growth and metastasis, while other advocate that cancer mitochondria exhibit impaired OXPHOS, and if this is fixed, cancer cells exhibit a smaller tumorigenic drive (Jia et al. 2019). Our grain of salt to this conundrum is to demonstrate that in some cancer cell lines OXPHOS activity and viability are uncorrelated; thus, a linkage of OXPHOS to cancer cell survival must be cell- and condition-defined (Doczi et al. 2022). Finally, mindful that during organism maturation it has been recently demonstrated that segregation of heteroplasmy is driven by the intracellular sorting of the organelles due to the impact of mtDNA on OXPHOS performance (Lechuga-Vieco et al. 2020), it is tempting to speculate that the same principles apply in tumor heterogeneity, i.e. some cells will 'inherit' mitochondria with sufficiently "good" mtDNA leading to high OXPHOS performance, while others will inherit mtDNA exhibiting a high number in mutations and/or copy number and demonstrate low OXPHOS performance; whether there is a correlation -if any- to tumorigenic potential, remains to be discovered.

Question 5. Are there any new data on the possible application of the substrate level mitochondrial phosphorylation system manipulations as a tool in tumor therapy, as promised in the dissertation?

>Response: Data on inhibiting mitochondrial substrate-level phosphorylation as a way of combating cancer remain unpublished and incomplete. When I was compiling the dissertation and before finalizing my publications records I was under the -false- impression that this work would be soon accepted. I did not wish to render such data public information prior to acceptance in peer-reviewed publications. An additional reason for withholding these data was to use them as preliminary results while applying for grants which I could not yet obtain (I have been applying since early 2021). Nonetheless, this is the progress on this aspect of my work: As there are no specific inhibitors for succinate-CoA ligase, we were left with the only option of either hindering the expression of mRNA coding for its subunits using siRNA technology, or knocking down (or out) the respective genes using CRISPR. Nowadays, inducible CRISPR systems are available, but we are not yet confident enough -i.e. we lack the molecular biology expertise- for applying them. When we were adding siRNA targeting mRNA coding for SUCLG1 or SUCLA2 to cancer cell lines (VM-M3, VM-M2), cells were unable to grow. Although this is a strong indication that the viability of these cancer cell lines critically depend on succinate-CoA ligase, we could not verify that the siRNA was targeting only succinate-CoA ligase mRNA as we could not collect any cells for proteins extraction. Furthermore, this treatment was not so successful on other cell lines such as BV-2, but there we encountered rather low transfection efficiencies. In addition, our means for recording viabilities of cells in monolayer cultures was not very robust, until recently. Now we are able to follow viability by high-throughput automated microscopy using two viability dyes, one for all cells and another for those with an impairment in plasma membrane integrity labeling dead or committed to die cells (Doczi, Karnok, Bui, Azarov, Pallag, Nazarian, Czumbel, Seyfried and Chinopoulos 2022). Finally, we are trying to secure funding for identifying high-affinity ligands for succinate-CoA ligase using DNA-encoded libraries (DEL). DELs consist of hundreds of thousands of chemical compounds or fragments conjugated to DNA sequences in a single vial. In this approach, our target (succinate-CoA ligase) is immobilized on an

artificial substrate and co-incubated with the DEL; those chemical compounds (or fragments) exhibiting a sufficiently high affinity to the target bind to it and are subsequently co-eluted; the high-affinity compounds are then identified through the conjugated DNA sequence using PCR and next generation sequencing. So far, we have been able to produce a catalytically active succinate-CoA ligase from recombinant protein subunits. We will be able to check if any high-affinity ligand(s) identified by the DEL method is/are inhibitory to succinate-CoA ligase using assays that we have also developed, and only then apply the compounds to cancer cell lines and test their effect on viability.

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March 01, 2023

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