Regarding the aim using SUCLA2 knock out mice, this transgenic animal is still on the process of being generated, even though it has been paid in full. After the generation of two successful clones, the company (TIGM) has moved into a new location, that back-logged everything for at least one year. As soon as the mice will arrive in our facility, it will be a top priority to perform all remaining proposed experiments.
Significant progress on two other transgenic mouse colonies, one being 25-45% deficient (depending on the gender of the mouse) for the E2 subunit (dihydrolipoyl succinyl transferase subunit (DLST; E.C. 2.3.1.61) of the alpha-ketoglutarate dehydrogenase complex and another being deficient for the E3 subunit (DLD, E.C. 1.8.1.4) of the alpha-ketoglutarate dehydrogenase complex, has been achieved. The alpha-ketoglutarate dehydrogenase complex is one step upstream from succinate thiokinase in the citric acid cycle, and it has a very large flux control coefficient. This means that a decrease in the complex' activity, must result in a decrease in all of the downstream products of the citric acid cycle. By comparing isolated mitochondria from the livers and brains of male and female DLD and DLST heterozygous (the homozygotes do not survive past embryonic day 10) versus wild-type mice, we found that mice exhibit a sufficiently decreased KGDHC flux to diminish ATP formation by SUCL-mediated substrate level phosphorylation to such a degree, that the hypothesis formed on the basis of results obtained from previous experiments is strongly supported. Regarding our laboratory findings with the DLD and DLST mice, these are detailed as follows: We isolated mitochondria from the brains and livers of WT and heterozygote littermate female animals for the DLST gene (DLST +/-) and DLD gene (DLD+/-), and performed similar experiments as described in FASEB J, 2010. Specifically, i) we measured membrane potential (Dpsim) in isolated mitochondria, and checked the effect of carboxyatractyloside (catr) versus oligomycin (olgm), specific ANT and ATPase inhibitors, respectively, and ii) we measured ADP-ATP exchange rates mediated by the ANT in isolated mitochondria from WT and heterozygote DLST +/- animals for an array of mitochondrial substrates. Subsequently, complex I or III, was inhibited by the specific inhibitors, rotenone or stigmatellin, respectively. Subsequent addition of catr caused a more significant hyperpolarization or less significant depolarization in mitochondria isolated from WT than from DLST +/- or DLD+/- animals. This implies that the ANT reversed more readily in mitochondria from DLST+/- and DLD+/- animals, which could be a direct result of the diminished succinyl-CoA provision, resulting in an impaired matrix substrate-level phosphorylation, favoring ANT reversal. In contrast, if oligomycin is added in lieu of catr that lead to an immediate depolarization implying ATPase reversal. In all substrate combinations tested, DLST +/- and in some substrate combinations tested, DLD +/- animals exhibited a significantly decreased ATP efflux rate compared to WT littermates, consistent with a decrease in substrate-level phosphorylation. The present material not only supports the notion of substrate-level phosphorylation as a crucial component of intramitochondrial ATP production that prevents the ANT from reversing, it also points out that manipulation of succinyl-CoA provision to succinyl-CoA synthetase has a significant impact in matrix substrate-level phosphorylation. Western blotting for SUCLA2 and measurements of succinate thiokinase activity is identical between WT and DLD mice, pointing to the notion that the decrease in substrate-level phosphorylation is a flux issue. Finally, we collected urine from the DLD +/- and WT littermate animals in order to seek the presence of organic acids that would verify a moderate to severe degree of secondary succinate thiokinase deficiency, due to diminished succinyl-CoA provision.

Regarding siRNA against SUCLA2 in COS-7 and HEK293 cells, we have tried 4 different sequences. At least two of them were supposed to identify the monkey SUCLA2

mRNA (for four different monkey species, since COS-7 cell lines originated from monkey kidney carcinoma), however, we have not observed any silencing.

Regarding measurements of cytosolic/nuclear ATP by cytosolic/nuclear targeted luciferases and using bioluminescence by externally added luciferin: after the submission of our proposal, a new plasmid has been generated that was supposed to report changes in cytosolic ATP/ADP ratios, by changing the fluorescent properties of a bacterial protein. We have obtained this plasmid from Dr. Garry Yelen, amplified it, and used it on both HEK293 and COS-7 cell. Our results show that the fluorescence spectra and quantum yield of this protein encoded by the plasmid significantly overlap with that of FADH2. Therefore, this plasmid is not suitable for our purposes.
11 additional plasmids have been generated by a Japanese group, shown to report cytosolic, nuclear, or mitochondrial ATP levels, operating as FRET-based indicators (Proc Natl Acad Sci U S A. 2009 Sep 15;106(37):15651-6); we have just obtained the plasmids, and we are currently expanding them in quantities.

Regarding aim (iii), using fibroblasts from patients suffering from SUCLA2 mutations, we used them as such without immortalizing them by inserting a telomerase gene. Our findings showed that -as predicted by the preliminary results- fibroblasts obtained from patients suffering from SUCLA2 mutations exhibited bongkrekic acid-induced depolarizations in respiration-impaired in situ mitochondria, implying premature ANT reversals. These findings have not been published yet, and we plan to submit them for publication together with the results obtained from the SUCLA2 transgenic mice.