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14. ABSTRACT

This project investigates the criticality of the mitochondrial phosphate carrier (PiC) for oxidative phosphorylation (oxphos; Aim 1) and buffering of mitochondrial matrix Ca^{2+} (Aim 2). Aim 3 focuses on the generation of TAT fusion proteins for the PiC and their ability to rescue phenotypes induced by PiC depletion. During this first year of the project there were accomplishments for each Aim. An accomplishment benefitting all Aims is the development of a new model, namely HEK293T cells with CRSIPR-cas9-mediated PiC knockout. The main accomplishments for Aim 1 were 1) finding that a hypothesized alternative Pi uptake pathway, namely the dicarboxylate transporter (DiC), does not compensate for PiC loss in HEK cells, 2) developing a tool to study the role of the DiC in isolated mouse skeletal muscle mitochondria, and 3) that, in opposition to a recent report in the literature showing indirect evidence that PiC is required for copper transport into mitochondria, we find that the PiC is not required for copper transport. Accomplishments towards Aim 2 are 1) the development of a robust experimental design to simultaneously measure mitochondrial matrix free Ca^{2+} as well as Ca^{2+} external to mitochondria in the HEK model and in isolated skeletal muscle mitochondria, and 2) the finding that matrix free Ca^{2+} concentration is elevated when PiC is depleted, supporting an important role for the PiC in matrix Ca^{2+} buffering. Finally, for Aim 3, TAT-PiCA has been generated and will soon be ready for testing in cells. Overall, this project is expected to 1) advance our basic knowledge about a fundamental process, namely how Pi is supplied to mitochondria for certain critical functions, and 2) to generate a potential therapeutic tool that might be useful in the management of a subset of human myopathies.

15. SUBJECT TERMS

Inorganic phosphate, Mitochondrial inner membrane transporters,Oxidative phosphorylation, Mitochondrial calcium uptake, TAT fusion protein, Mitochondrial myopathy, Mitochondrial disease, Nutrient signaling in skeletal muscle

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Section 3 – Accomplishments

Original Aims

Aim 1: To evaluate if PiC is critically required to transport inorganic phosphate into the mitochondrial matrix, and mechanisms that counteract PiC deficiency.
Aim 2: To test if PiC deficiency causes dysregulation of cytoplasmic and mitochondrial Ca^{2+} and Ca^{2+} regulated functions.
Aim 3: To generate TAT fusions proteins of PiC and to test if TAT-PiC can be delivered to cells.

The following describes only components from the Statement of Work that were anticipated to be completed during Year 1. Also included are parts of the project for which progress was made this year but had longer timelines than Year 1. A detailed description of the accomplishments follows.

Aim 1: The following had a longer timeline, but progress has been made during Year 1: Understanding alternate Pi transport mechanisms.
Aim 2: Anticipated Milestones for Year 1: Human primary muscle cells: calcium flux studies (cytosolic and mitochondrial Ca^{2+}), western blot analysis and qPCR (mitochondrial Ca^{2+} uptake machinery: **Completed/In Progress, however note a change in the plans:** We have developed lines of HEK cells with knockout of PiC using CRISPR-cas9. This was done because we anticipated that using cell lines would result in more robust data than using primary cells, both in terms of understanding the role of the PiC for mitochondrial Ca^{2+} uptake (Aim 2) and for testing the TAT-PiC proteins (Aim 3). Several months were needed to generate these knockout lines. For TAT-PiC testing, our goal was to generate several robust outcome measures. To that end, using the new HEK models we established 1) bioenergetics assays to evaluate oxidative phosphorylation and electron transport chain capacity and 2) cell proliferation protocols under culture conditions where mitochondrial ATP production can be minimized (glucose-containing medium) and where mitochondrial ATP production is crucial (glucose-devoid medium).

In addition, the following from Aim 2 was originally planned for Year 2, but progress on experimental protocol development was made this year: Ca^{2+} uptake studies in isolated skeletal muscle mitochondria.

Aim 3: Anticipated Milestones for Year 1: TAT-PiCA has been cloned and confirmed by enzyme restriction and sequencing analyses. TAT-PiCA has been isolated, and purification is in progress. Tasks that were completed: 1) To design and construct both TAT-PiC-A and TAT-PiC-B isoforms. Choosing the bacterial host for the expression of the TAT-PiCA/B fusion proteins. 2) Calibrating the conditions for high expression levels of the fusion proteins in a bacterial host. 3) Purification of the TAT-PiCA fusion proteins (in progress). The following closely associated tasks were anticipated to be completed in Year 1 but remain in progress or will be done during Year 1: 1) Testing correct targeting in cell cultures (immortalized MEFs and myoblasts as well as HEK-PiC(-/-) cells): To be done in Year 2. 2) Testing correct targeting in mitochondria isolated from mice with SkM PiC loss: To be done in Year 2. 3) Labeling fusion proteins with fluorescent dye (FITC) and testing its localization into mitochondria: To be done in Year 2

Accomplishments:

Section 1. Model systems (all Aims): Changes and Accomplishments

We originally proposed to use the following models in the Aims:

1) Vertebrate animal models. Mice with PiC depletion in skeletal muscle induced in adults by injection of tamoxifen.

2) Cell culture models: Muscle cells derived from myoblasts obtained from muscle biopsy from patients with loss of protein mutations in SLC25A3 encoding PiC (we have banked myoblasts that are de-identified), and from mice with PiC depletion.

We have not substantially changed our experimental model strategy but have made the below-described additions. This information is also described in the "Changes/Problems" section, and has been included here for coherence.

1) Vertebrate animal models: In addition to mice with tamoxifen(Tam)-induced adult-onset PiC loss only in skeletal muscle, we will include mice with *constitutive* PiC loss in skeletal muscle. The reason for the addition arose from a breeding problem in the Tam-induced adult-onset PiC loss colony. To stabilize the Tam-induced model, we are essentially re-deriving the model using new HSA-MCM (Human Skeletal Actin-MerCreMer) transgenic mice from Jackson Laboratories (purchase using non-DOD funding). The HSA-MCM mice carry a transgene that drives skeletal muscle-specific Cre recombinase expression induced by Tam. These mice are currently being bred to $PiC^{fl/fl}$ mice; the new breeding pairs have thus far been productive. We also obtained mice harboring a constitutive HSA-Cre transgene that we bred with $PiC^{fl/fl}$ mice to provide mice with constitutive PiC depletion in skeletal muscle; these mice are breeding well. Thus we will be able to compare mice with short term and longer term PiC depletion. We expect to have sufficient numbers of HSA-Cre-PiC mice for experimental groups in a couple of months and to have sufficient numbers of HSA-MCM-PiC in ~8 months. We purchased mice and (re)derived these colonies under another IACUC protocol and using non-DOD funds.

2) Cell culture models: We have generated HEK293 cells with knockout of SLC25A3 using CRISPR-cas9. This model was developed because HEK cells are easy to grow, maintain and transfect as compared to primary muscle cells. Thus the HEK cells would provide us with a "workhorse" model. HEK cells are far less physiologically relevant than muscle cells, and, as proliferating rather than differentiated cells, are fundamentally different from muscle cells. However, understanding the impact of PiC on mitochondrial and cytoplasmic Ca^{2+} dynamics (Aim 2) and testing TAT-PiC proteins (Aim 3) can certainly be substantially evaluated, at least initially, in the simpler model offered by the HEKs. The following describes the generation and testing of the HEK model, which we consider to be an **accomplishment of Year 1**. The HEK model was generated by post-doctoral fellow, Dr. Valentina DeBattisti.

Generation of HEK cells with knockout (KO) of SLC25C3 encoding PiC using CRISPR-cas9.

We first generated polyclonal PiC-KO and Empty (EV)-Vector cells according to the following workflow:

1) Six sgRNAs were designed that target exon3 of the human SLC25A3 gene using the web-based tool www.genome-engineering.org (Hsu et al, 2013) that evaluates off-target effects and the Genetic Perturbation Platform http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design (Doench et al, 2014) that evaluates efficacy.

2) The sgRNA were then cloned into the LentiCRISPRv2 backbone. Five of 6 sgRNAs were successfully cloned based on sequencing (Figure 1).

3) SLC25A3 sgRNA combinations #1+#2 and #5+#6 that were cloned into the LentiCRISPRv2 backbone were transiently transfected into HEK293T cells using polyethylenimine.

4) Forty eight hrs after transfection, cells were selected for 72 hrs using puromycin.

5) After selection, an aliquot of cells was collected for genotyping.

6) Aliquots of the cells were frozen. Other aliquots of some cells were to generate monoclonal PiC-KO and EV control cells.

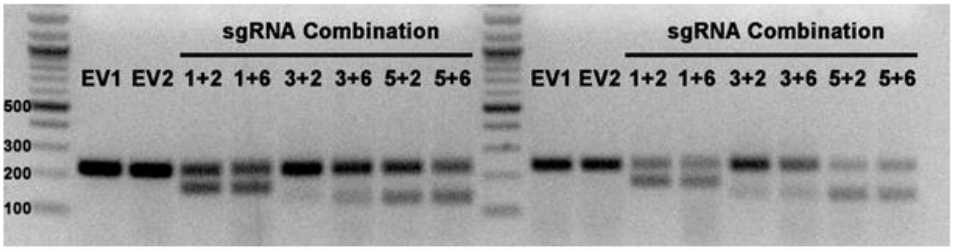


Figure 1. Genotyping (PCR) for polyclonal HEK-PiC-KO and empty vector (EV) lines generated using different combinations of CRISPR guide-RNAs (gRNA). Upper band is the wild-type, lower band shows incorporated gRNAs. The gRNA combinations 1+2 and 5+6 were used for generating monoclonal lines

We next generated monoclonal PiC-KO and EV cells according to the following workflow:

1) Cells were counted using at least two unique samples to improve count accuracy.

2) Cells were diluted into 30ml of media at a concentration of 2.5cells/ml.

3) The dilute cell suspension was aliquoted at 100ul/well into a 96 well plate. Thus, at 2.5cell/ml, 100ul/well provided for 0.25cell/well or 1cell/4wells; this improved the possibility of obtaining colonies derived from a single cell.

4) After 2 weeks, colonies were observed in some wells. At 60-80% confluency, colonies were passaged into a 24-well plate. A 20-ul aliquot was retained for genotyping (Figure 2). shows genotyping results from colonies that grew in the 96-well plate.

5) Figure 3 shows immunoblot analysis of several clones and controls that were able to grow. Some colonies did not proliferate well in the 24-well plate thus could not be used.

6) K06, K09, K011, K028 and several EV controls all grew in 24-well plates and have been, or will be, used in subsequent analyses. Aliquots of cells were frozen for subsequent analysis (see below).

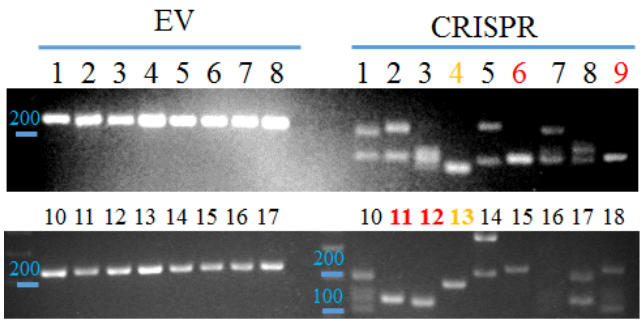


Figure 2. Genotyping (PCR) of monoclonal HEK lines. Upper band shows wild-type PiC. Lower band shows PiC KO. Presence of two bands indicates a polyclonal population. Presence of a single lower band suggests PiC KO (clone number in black). CRISPR clone number in orange: inconclusive. Clone number in red: selected as monoclonal lines for study. EV: empty vector.

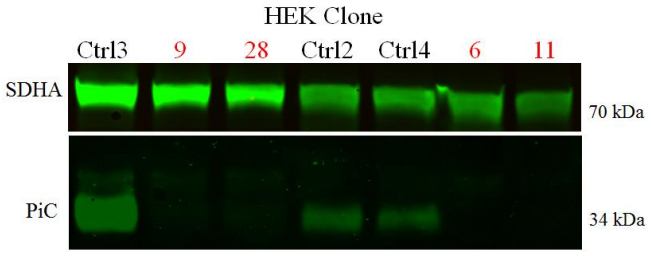


Figure 3. Immunoblot analysis of PiC expression in empty vector control HEK lines (Ctrl) and knockout lines (numbers in red). SDHA: succinate dehydrogenase, used as loading control. Whole cell lysates were used; 50ug/lane.

Accomplishment: Thus, a major accomplishment of Year 1 is the generation of several clones of HEK-PiC-KO cells and EV Controls. This model is expected to be useful for all Aims, as described below.

Section 2. Aim 1 Accomplishments

Bioenergetic and growth analyses in HEK-PiC-KO cells.

2.1. Rationale and Goal

Our goal was to establish the growth and mitochondrial bioenergetics phenotypes of HEK-PiC-KO clones, in order to 1) address the primary hypothesis of Aim 1, namely that PiC is

critical for Pi transport into mitochondria to sustain oxidative phosphorylation (oxphos) and thus cell proliferation, and 2) establish robust phenotypes on which to test TAT-PiC fusion proteins developed in Aim 3. These studies have been and will continue to be conducted by first year Ph.D. student Ariana Entezari (first year tuition and stipend covered by the University, as is customary).

2.2. Approach and Methods

Generally, we followed protocols established in our published work on patient fibroblasts with novel compound heterozygous SLC25A3 mutations and HeLa cells with acute PiC knockdown using siRNA (Seifert et al., 2016).

Growth curves were generated by seeding cells at ~200k/well into 6-well dishes, then trypsinizing and counting cells after 24, 48 and 72 hrs. Two culture medium conditions were used: 1) regular DMEM with 25 mM glucose and 4 mM glutamine which can sustain growth even in cells with defective oxphos because glucose is abundantly available for glycolysis, 2) DMEM devoid of glucose but containing amino acids including glutamine, as well as 3 mM β-hydroxybutyrate; this creates a dependence on mitochondria for ATP synthesis. Note that in both conditions, and during maintenance culturing, culture medium was supplemented with uridine to offset any depletion of UMP (uridine monophosphate, used for RNA synthesis) caused by a defective electron transport chain. Duplicate wells were used for each experiment, and experiments on a particular clone were conducted in at least 2 passages of that clone.

Mitochondrial bioenergetics was analyzed using a Seahorse XF24 Flux Analyzer in permeabilized cells in order to directly test for an inorganic phosphate (Pi) limitation without the complexities of oxphos regulation due to factors outside of mitochondria. The focus was on 2 different substrate conditions, pyruvate/malate and succinate(plus rotenone), because these substrates gave highly informative results in preliminary studies using skeletal muscle mitochondria isolated from mice with PiC depletion only in skeletal muscle. Specifically, we observed that isolated skeletal muscle mitochondria with PiC loss supplied with succinate(plus rotenone) had normal leak-dependent respiration (induced by

oligomycin that inhibits the ATP synthase thereby allowing a small amount of proton return via pathways other than the ATP synthase) and normal or elevated maximal electron transport chain activity (induced by the chemical uncoupler FCCP) but were unable to perform oxphos. In stark contrast, supplying these mitochondria with pyruvate/malate resulted in oxphos that was ~20-30% of that measured in mitochondrial from control mice. This provide initial evidence for alternative Pi uptake by the dicarboxylate transporter, DiC (encoded by SLC25A10) and which can be inhibited by butylmalonate. Thus, in HEK experiments we also established the use of butylmalonate to test for the potential involvement of the DiC to transport Pi into PiC-depleted HEK cells. Additionally, because use of the DiC to transport Pi into the mitochondrial matrix would also involve the loss of dicarboxylates (namely malate and succinate) in exchange for Pi, we devised protocols to test for the role of the Krebs cycle. We reasoned that stimulation of oxphos by means that are independent of the Krebs could be used to test if Krebs cycle intermediates (namely succinate and malate) are needed for oxphos in PiC depleted HEK cells. To this end, bioenergetics experiments were also conducted using the electron donor duroquinol (plus rotenone to inhibit Complex I: duroquinol+rotenone will cause electrons to enter the electron transport chain at Complex III, without any involvement of the Krebs cycle) and TMPD/ascorbate (plus antimycin to inhibit Complex IV: TMPD/ascorbate+antimycin will cause electrons to enter the electron transport chain at Complex IV, without any involvement of upstream process including the Krebs cycle). Thus we have devised 3 tests of the involvement of the DiC. Furthermore, use of TMPD/ascorbate also tests the hypothesis that the PiC functions as a copper transporter, which was recently suggested (Boulet et al., 2018). Technical replicates of 3 – 5 were used per passages, and at least 2 passages of each clone were tested (except where indicated).

Follow-up qPCR and immunoblot analysis will be performed on samples of each culture from an early passage (usually P4) and from a later passage (P8-11) in order to determine if KO clones are indeed completely devoid of PiC. Discussions with colleagues including our Haya Lorberboum-Galski, a sub-awardee on this grant, indicate that, despite best practices, clones many not be monoclonal. Thus, we need to determine if low level expression of PiC protein (which would likely increase with passage number of cell

expressing low level PiC have a growth advantage) accounts for major variations in phenotype among the "KO" clones.

Analysis: Different EV control lines will be averaged to generate the "EV Control" phenotype. For each KO clone, data from the 2 passages will be averaged to obtain the phenotype for that KO clone. Eventually we will have data from all 4 KO clones. Preliminary analysis will compare bioenergetics and growth phenotypes versus SLC25A3 mRNA and PiC protein expression. Eventually all "KO" clones can be used to address the Aim 1 hypothesis; in other words, we can take advantage of variable expression levels of PiC if this is what occurs. On the other hand, HEK cells to be used for TAT-PiC protein testing (Aim 3) should have a substantial phenotype sustained across several passages in order to have a robust test system for the TAT-PiC proteins. Moreover, we hope to have more than one KO clone having a similar phenotype in which to test the TAT-PiC proteins. The aforementioned describes only the preliminary analysis that will be applied to this phase of the project. Regarding bioenergetics, all O2 consumption rates are reported after subtraction of any antimycin-insensitive rate (a measure of non-mitochondrial O2 consumption); antimycin-insensitive rates were usually minimal in permeabilized cells.

2.3. Results and Discussion

All PiC-KO and EV-Control (Ctrl) lines grew well in regular DMEM (25 mM glucose, 4 mM glutamine, supplemented with uridine), and cells did not display unusual morphologies. Table 1 summarizes the proliferation rate and bioenergetics ("JO2": O2 flux) experiments conducted thus far. Remaining experiments in this series are expected to be completed by the end of November 2018.

Cell proliferation rate. The cell proliferation was evaluated as a complex readout of ATP availability and over cell fitness. Figure 4 shows an example of delayed growth in HEK-PiC-KO cells compared to EV-Control cells in both glucose containing medium and in medium containing only mitochondrial (mito) substrates. Delayed growth was apparent in KO6 and KO9, similar to what we observed previously in HeLa cells with acute knockdown of PiC using siRNA, whether by ~65% or ~85% (Seifert et al., 2016). It was

Table 1: Proliferation rate and bioenergetics (JO2) studies completed by October 2018, with summarized results. ↓: decrease relative to EV-Ctrl; =: no change from EV-Ctrl (EV-Ctrl3 and EV-Ctrl4).

HEK CRISPR Clones:	**KO6**	**KO9**	**KO11**	**KO28**
Proliferation rate, glucose	↓	↓	=	
Proliferation rate, mito substrates	↓	↓	=	
JO2, pyruvate/malate		=	=	=
JO2, succinate/rotenone		=	=	=
JO2, duroquinol/rotenone		=	=	
JO2, TMPD/ascorbate/antimycin		=	=	

Empty boxes: Experiments to be completed by the end of November, 2018.

interesting to note that delayed growth in those clones was similar in either media condition; in other words, proliferation did not worsen in medium that provoked ATP synthesis from mitochondria. Thus, the cause of slower proliferation is not simply decreased mitochondrial ATP synthesis. Subsequent studies will address if apparent slower cell proliferation in KO6 and KO9 reflects increased cell death. In contrast to KO6 and KO9, the KO11 clone proliferated at a similar or faster rate as compared to EV-Ctrl cells. Table 2 summarizes the doubling time calculated in the HEK clones.

Table 2: Slower doubling time (hrs) of some HEK CRISPR KO clones:
Values: mean ± standard deviation (n=2 passages per clone; 2 technical replicates/passage).

HEK Clones:	**EV-Ctrl3**	**EV-Ctrl4**	**KO6**	**KO9**	**KO11**	**KO28**
Doubling time (hrs), glucose	15.2 ± 0.3	14.9 ± 1.5	18.6 ± 0.4	20.5 ± 0.6	14.7 ± 0.8	NA
Doubling time (hrs), mito substrates	22.4 ± 3.6	19.8 ± 1.6	25.6 ± 1.3	NA	19.7 ± 3.5	NA

NA: Not available.

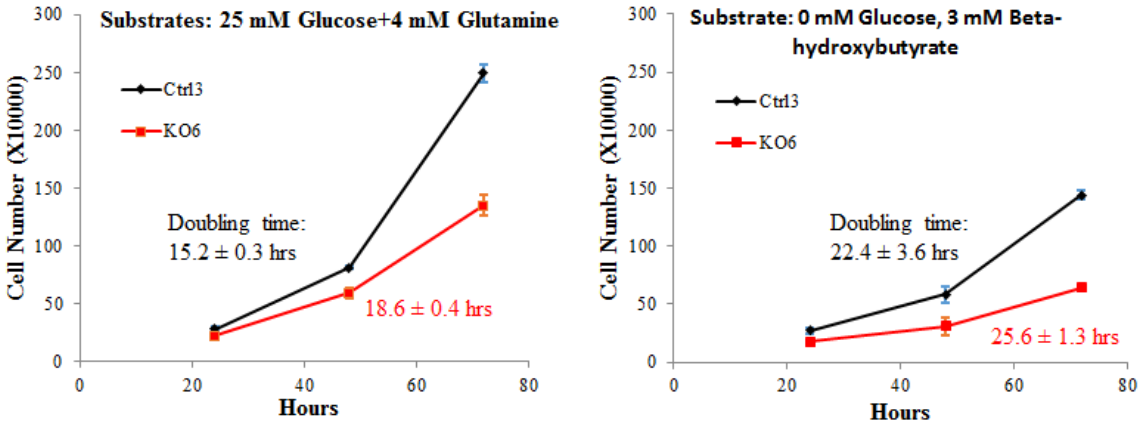


Figure 4. Cell proliferation is slower in some HEK-PiC-KO clones, shown here for HEK-PiC-KO clone KO6 and empty vector (EV) Ctrl3. Cells were grown in either glucose-containing medium that favors ATP synthesis by glycolysis or medium devoid of glucose and containing substrates that can only be used by mitochondria for ATP synthesis. Cell were counted 24, 48 and 72 hrs after seeding. Symbols are mean ± standard deviation (2 passages, 2 technical replicates for each time point, per passage). Doubling time in hours for each group is shown next to the growth curve.

Bioenergetics. Mitochondrial bioenergetics was evaluated because the hypothesized defect caused by PiC depletion was a major limitation on oxphos, with no limitation on leak-dependent O2 consumption (measured as oligomycin-insensitive O2 consumption; oligomycin inhibits the ATP synthase) and no limitation in maximal uncoupled O2 consumption (measured as O2 consumption in the presence of FCCP, a chemical uncoupler of oxidation and phosphorylation). As explained in Methods, different substrates were used because these yielded insight into mechanisms for alternative Pi transport in mitochondria isolated from mouse skeletal muscle depleted of PiC (not shown).

To evaluate mitochondrial bioenergetics, we followed protocols to test for Pi limitation of oxphos in HEK-PiC-KO cells that were based on protocols demonstrating that acute depletion of PiC, using 2 different siRNA duplexes, limited maximal oxphos by 30-50% when PiC was depleted by ~85% in permeabilized HeLa cells (Seifert et al., 2016). Interestingly, HeLa cells with ~65% PiC depletion had similar maximal oxphos as Control cells (Seifert, 2016). We therefore concluded that PiC is normally expressed in great excess of the requirement of maximal oxphos, at least in HeLa cells.

We conducted bioenergetics determinations in HEK cells permeabilized as in we have done previously (Seifert et al., 2016). Using our established quality control measures, we deemed all recordings as were stable, with linear O2 traces, even after injection of oligomycin (to inhibit the ATP synthase and thus to reveal leak-dependent O2 consumption); thus all data were of high quality and interpretable, as in our previous study (Seifert et al., 2016). An example of O2 traces from a typical experiment (EV-Ctrl4 *vs.* KO11), together with analyzed data, for duroquinol(+rotenone) as the substrate, is shown in Figure 5.

As summarized in Table 1, and as shown in an example of data from HEK-PiC-KO9 (Figure 5), we observed no changes in any of the bioenergetics parameters that we measured. This is in stark contrast to what we observed in permeabilized HeLa cells with acute PiC depletion by 85% (Seifert et al., 2016). HeLa cells with acute PiC depletion by ~65% or primary patient fibroblasts harboring compound heterozygous PiC mutations resulting in ~60% loss of PiC protein failed to show differences in maximal oxphos as determined in permeabilized cells. These studies suggest that some the KO9, KO11 and KO28 clones contain residual PiC protein and/or there is a sufficiently high capacity alternative route for Pi uptake into mitochondria that (substantially) lack PiC. We note that oxphos was equally unimpaired under each of the substrate conditions, including duroquinol and TMPD/ascorbate that bypassed the Krebs cycle and thus would impair Pi uptake via the DiC if that were operating as the alternate Pi transport mechanism.

Therefore, it is unlikely that the HEK clones with substantial PiC loss rely on the DiC for Pi transport into the matrix.

It should be noted that it is not unusual that some KO clones proliferated at a slower rate despite the lack of any obvious restriction on maximal oxphos. Indeed, this was also observed in HeLa cells with acute 65% PiC depletion and the aforementioned patient

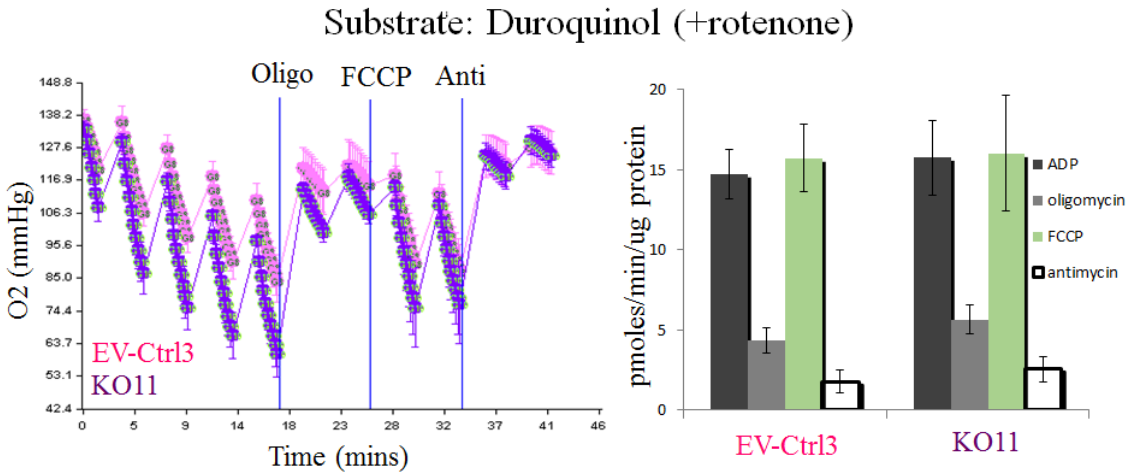


Figure 5. Bioenergetics evaluation, by measuring O2 consumption (JO2), of HEK-PiC-KO and empty vector (EV) control (Ctrl) cells. Cells were permeabilized; thus JO2 mainly reflected that from mitochondria. Saturating ADP and substrate (duroquinol) were added to permeabilized cells from the start of the experiment; the resulting JO2 reflected maximal oxidative phosphorylation (i.e., ATP synthesis from mitochondria). Oligomycin (inhibitor of the ATP synthase) was injected to determine the maximal non-phosphorylating JO2 which is important to determine because it can be substantial in some cases and offset oxidative phosphorylation. The chemical uncoupler FCCP was injected to evaluate maximal electron transport chain activity. Finally, antimycin A (inhibitor of Complex III of the electron transport chain), was used to evaluate non-mitochondrial JO2. Representative data are shown for HEK-PiC-KO clone KO11 and control EV-Ctrl3. Left panel: raw O2 traces. Right panel: averages ± standard deviation for 3-4 technical replicates; these values were normalized to total cellular protein (averaged from the technical replicates).

fibroblasts (Seifert et al., 2016). Intact patient fibroblasts displayed a suppressed basal oxphos that likely reflected either a stress response or lesser ATP demand, possibly as an adaptation to less PiC protein or to mitochondrial stress triggered by misfolded PiC proteins (Seifert et al., 2016). In addition to completing bioenergetics studies in permeabilized KO6 cells, we will undertake bioenergetics studies in intact KO EV-Ctrl clones to address the

possibility that basal bioenergetics is indirectly altered by cell stress due to PiC depletion, leading to slowed cell proliferation.

To further understand, we overexposed the immunoblots shown in Figure 3. Figure 6 (over-exposed blots from Figure 3) reveal possible low level expression of PiC protein in KO9, KO11 and KO28. KO6 may have less or no expression of PiC. We have also quantitatively evaluated mRNA expression by qPCR analysis. We designed primers specific for both

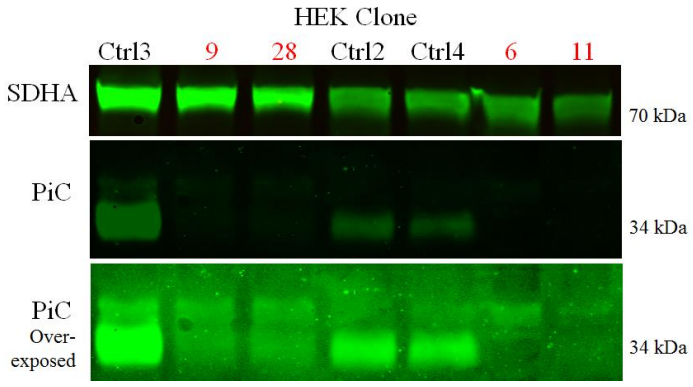


Figure 6. Immunoblot from Figure 3, but with over-exposure of the PiC signal. Over-exposure reveal possible residual protein in HEK-PiC-KO clones KO9, KO11 and KO28.

PiC-A and PiC-B isoforms; only PiC-B would is expected to be expressed in HEK cells since PiC-A is only expressed in skeletal and cardiac muscle. Indeed, primers for PiC-B revealed strong expression in EV-Ctrl cells whereas PiC-A was essentially undetectable in EV-Ctrl cells. We also determined that the efficiency for the primers for both PiC isoforms were similar; thus, this allows us to directly compare the Ct values obtained for PiC-A and PiC-B. Importantly, this provides us with Ct values that correspond to undetectable mRNA. While the Ct values for PiC-B in KO9, KO11 and KO28 clones were much higher (corresponding to not more than 10% of EV-Ctrl PiC-B mRNA levels), those Ct values were still lower than for PiC-A; in other words, it seems unlikely that KO9, KO11 and KO28 have complete PiC knockout. Furthermore, mRNA analyses suggested more residual PiC mRNA in KO11 as compared to KO9 and KO28, which is in line with the proliferation rate of these cells that was similar to that for EV-Ctrl cells. KO6 mRNA will soon be tested.

As mentioned above, we experimented with the DiC inhibitor butylmalonate (BM) as one means to test the role of the DiC in the context of PiC loss. Using HEK EV-Ctrl cells we determined that co-addition of BM together with substrate to permeabilized cells required a lower concentration of BM to inhibit pyruvate/malate or succinate(plus rotenone) respiration as compared to when BM was added to permeabilized cells already respiring on the aforementioned substrates. This indicated that BM was competing with substrate for transport across the DiC. We were also able to determine effective concentrations for inhibition of respiration. However, we found that even low doses of BM inhibited maximal oxphos in EV-Ctrl cells respiring on either pyruvate/malate or succinate(plus rotenone). This actually meant that maximal oxphos in EV-Ctrl cells crucially depended on the DiC for malate and succinate uptake. Thus, we concluded that BM was not a useful tool to investigate the role of the DiC in PiC-depleted HEK cells.

On the other hand, we have now determined BM to be a useful tool to study the role of the DiC in skeletal muscle mitochondria isolated from mice with PiC depletion in skeletal muscle. Mitochondria were harvested from mice bred and sacrificed on another IACUC protocol. Highly functional mitochondria were isolated from skeletal muscle according to our established protocol (Seifert et al., 2008). Bioenergetics were measured in isolated mitochondria using the Seahorse XF24 instrument, generally following our previous protocols (Moffat et al., 2014). We determined in skeletal muscle mitochondria from Ctrl mice that 5 mM of BM fully inhibits succinate-driven maximal oxphos while leaving pyruvate/malate-driven maximal oxphos intact (data not shown). The latter was evaluated through dose-response analysis of mitochondria respiring on the above-mentioned substrates, and was repeated on mitochondria isolated from 3 Ctrl mice, yielding consistent results. Thus we have developed a new tool to evaluate the importance of the DiC (specifically, the DiC driven by succinate transport) in PiC-depleted skeletal muscle mitochondria respiring on pyruvate/malate without directly affecting pyruvate/malate-driven maximal oxphos.

Accomplishment: In conclusion, low levels of PiC protein, likely not more than 10% of the levels in EV-Ctrl and possibly less than 5% of the level in Ctrl cells, may fully or

partially account for the sustained maximal oxphos in the HEK-PiC-KO clones tested thus far. It is possible that alternative these KO clones are further supported by Pi uptake across another transporter; however, our data suggest that this alternate transport mechanism is not the DiC, at least in HEK cells. The DiC may be an alternate Pi transport in skeletal muscle mitochondria depleted of PiC, and we have the developed the use of BM to test this possibility. Overall, the results from the bioenergetics analyses of the HEK-PiC-KO clones generally mirror our observations in HeLa cells with acute PiC knockdown and patient fibroblasts with partial PiC knockdown, and allow us to conclude that very little PiC, possibly as little as 5%, is needed to sustain normal maximal oxphos, at least in cultured cells. Furthermore, sustained TMPD/ascorbate oxphos in HEK-KO cells indicates that, if PiC is required for copper transport into mitochondria as was suggested (Boulet et al., 2018), then very low PiC expression is sufficient for the copper needs of mitochondria.

Next steps: For reasons detailed in Section 3.3, below, it is of interest to evaluate the bioenergetics phenotype in intact HEK-PiC-KO in the context of protocols that elicit mitochondrial Ca^{2+} uptake; this will allow us to determine if low levels of Pi uptake result in a prioritization of the Pi for ATP synthesis over buffering of matrix Ca^{2+}, or *vice versa*. These experiments would also allow us to address whether basal oxphos in intact cells is restricted, which might explain the slower proliferation rate in some PiC-KO clones. Once our IACUC and ACURO protocols are accepted, we will begin a systematic investigation of the bioenergetics and also Pi transport in isolated skeletal muscle mitochondria from Control and skeletal muscle-specific PiC-depleted mice. Studies will also be initiated to understand the impact of PiC depletion in skeletal muscle on skeletal muscle force generation and fatiguability, to be studied in the lab of Dr. Tejvir Khurana.

Section 3: Aim 2 Accomplishments

3.1. Rationale and Goal.

PiC-mediated Pi influx is coupled to mitochondrial Ca^{2+} accumulation and likely affects both the amount of Ca^{2+} uptake into the mitochondrial and Ca^{2+}-binding (i.e., buffering) in the matrix. Our goal was to determine if mitochondrial Ca^{2+} transport and matrix Ca^{2+} buffering depend on Pi uptake via the PiC. These studies were conducted by post-doctoral

fellow, Dr. Valentina DeBattisti, and will continue to be conducted by Research Assistant Aish Sivaramakrishnan.

3.2. Approach and Methods

Model: The HEK-PiC-KO and EV lines were used as model systems. Preliminary experiments were also conducted in isolated skeletal muscle mitochondria from mice, as described; please note that mice were bred and sacrificed under another IACUC protocol.

Overall approach for evaluating Ca^{2+} in intact cells. The concentration of Ca^{2+} that is free in the mitochondrial matrix ($[Ca^{2+}]m$) will be monitored simultaneously with the concentration of Ca^{2+} in the cytoplasm $[Ca^{2+}]c$ using genetically targeted Ca^{2+} sensitive fluorescent protein (mtCepia to measure $[Ca^{2+}]m$; Ex 480 nm/Em 525 nm, Kd 13 µM) or dye (Fura2-AM to measure $[Ca^{2+}]c$; Ex 490nm/Em 515nm, Kd 4.5 µM) and microscopic imaging in intact and also eventually in permeabilized cells, as described in recent studies from the Hajnóczky lab (e.g., Csordas et al., 2013). This approach quantifies amount of Ca^{2+} accumulated by mitochondria and the corresponding change in free Ca^{2+} in the matrix. Thus, Ca^{2+} chelation in the matrix ("buffering") can be directly compared in genetically targeted models and the relevant control, and as a function of the amount of Ca^{2+} taken up which is measured as Ca^{2+} clearance from the cytoplasm.

For cell imaging experiments, the cells were pre-incubated in a serum-free extracellular medium (ECM, 121 mM NaCl, 5 mM $NaHCO_3$, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM $MgSO_4$, 10 mM glucose, pH7.4) containing 2% BSA. $CaCl_2$ was omitted from the ECM. Fluorescence wide field live cell imaging of $[Ca^{2+}]c$ and $[Ca^{2+}]m$ was carried out using a ProEM1024 EM-CCD (Princeton Instruments), fitted to Leica DMI 6000B inverted epifluorescence microscopes. Images were obtained in every 2s. The fura2 ratios were calibrated in terms of nM $[Ca^{2+}]c$, whereas the mtCepia fluorescence at each time point was normalized to the initial fluorescence (F0/F).

Newly devised approach for evaluating Ca^{2+} in isolated mitochondria. While it is fairly routine in isolated mitochondria suspensions to monitor Ca^{2+} concentration in the buffer

[Ca^{2+}]b, it is less straightforward to monitor [Ca^{2+}]m. To measure [Ca2+]m in isolated mitochondria, mitochondria were loaded with Fura-FF-AM (Ex 490nm/Em 515, Kd 4.5 µM) in isolation medium containing 0.3% pluronic acid at 35ºC for 30 minutes. Mitochondria were then pelleted at 9000 g for 10 minutes at 4ºC. Mitochondria were then depleted of Pi so that mitochondria from both genotypes (Ctrl and PiC-depleted) started with zero Pi in the matrix. To deplete mitochondria of Pi, mitochondria were suspended in isolation medium containing 0.75 units/ml of hexokinase, 1 mM glucose, 0.5 mM ADP, 1 mM $MgCl_2$ and pyruvate/malate (5/5 mM), for 10 mins. Mitochondria were then spun again at 9000 g for 10 minutes at 4ºC and suspended again isolation buffer with no additions. Mitochondria loaded with Fura-FF-AM and depleted of Pi were found to generate a robust membrane upon addition of substrate (pyruvate/malate or succinate) that was similar to mitochondria that had not undergone these procedures (not shown). [Ca^{2+}]b was measured using Rhodamine-2-FF (Ex 550nm/Em 570, Kd 19 µM).

Experimental design details are provided in the Results and Discussion section that follows.

3.3. Results and Discussion

Establishing the approach to induce mitochondrial Ca^{2+} uptake. A first goal was methodological and it was to determine how best to stimulate the uptake of mitochondrial Ca^{2+} by the mitochondrial Ca^{2+} uniporter (mtCU) in intact cells. This can be done either through agonist stimulation leading to Ca^{2+} release from the endoplasmic reticulum (ER) via IP3 receptors (IP3R) or via store operate Ca^{2+} entry (SOCE). We first attempted agonist stimulation using ATP; however, this induced little uptake of Ca^{2+} into mitochondria (not shown). Separate studies underway in the Hajnóczky have since determined that in HEK cells there is relatively loose coupling between ER and mitochondria, thus much of the Ca^{2+} released from IP3R would more likely diffuse into the cytoplasm leading to little Ca^{2+} in the vicinity of mitochondria. Thus conditions were set up to induce mitochondria Ca^{2+} uptake using a SOCE protocol. To induce SOCE, prior to experiments the ER was depletion of Ca^{2+} stores by a 10-minute incubation in 2 µM thapsigargin as in (Csordas et al., 2013). To evoke SOCE, 2 mM was used as a relatively physiological stimulus, and 10

mM was also to force strong Ca^{2+} entry in cells where store-operated Ca^{2+} entry is not strong.

These above-described methods were found to be robust. Our protocol was to run study, on each experimental day, cells from EV-Ctrl (EV-Ctrl4) and from 2 PiC-KO clones (KO9 and KO28). An experimental day consisted of imaging usually > 20 cells/genotype, measuring [Ca2+]c and [Ca^{2+}]m. In total there were 5 successful experimental days (representing different passages of the cells) where at least adequate quality data was obtained from many >20 cells from each of the 3 lines. Figure 7 shows representative traces from all 3 genotypes, for both the 2 mM and 10 mM SOCE protocols. Qualitatively similar results were obtained on 3 of the other 4 experimental days. Quantification of data from all experimental days are in progress. What Figure 7 clearly shows is that in both KO clones compared to the EV-Ctrl, the rise in [Ca^{2+}]m occurred at lower cytoplasmic Ca^{2+} in the

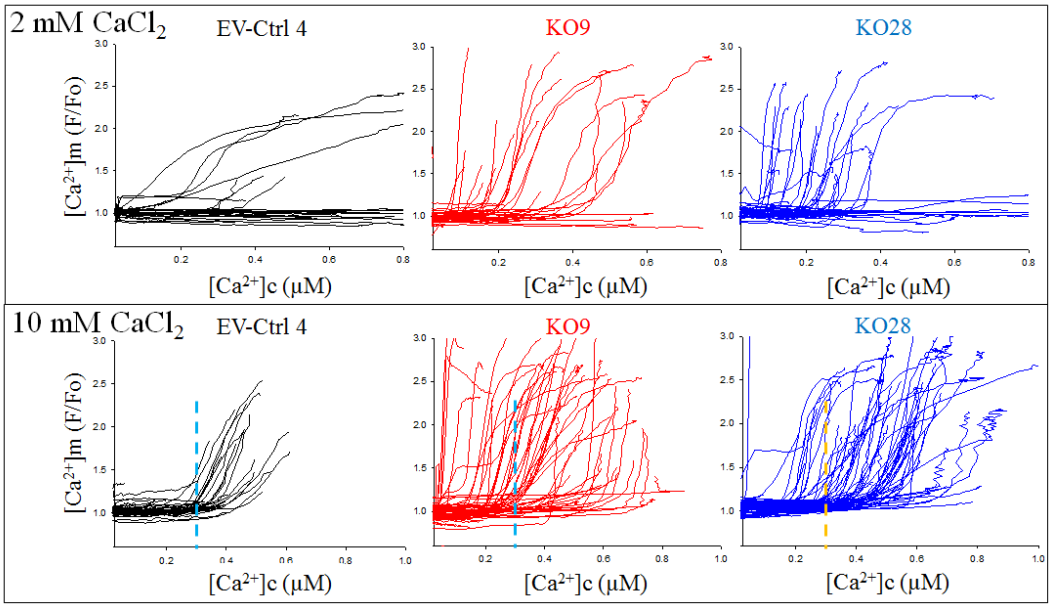


Figure 7. Plots of cytosolic Ca^{2+} concentration [Ca^{2+}]c versus mitochondria matrix free Ca^{2+} concentration [Ca^{2+}]m after exposing intact HEK-PiC-KO lines (KO9 and KO28) and empty vector (EV) controls (Ctrl4) to 2 or 10 mM $CaCl_2$. Before experiments, cells were treated with thapsigargin to deplete the endoplasmic reticulum Ca^{2+} stores; thus [Ca^{2+}]c reflected store operated Ca^{2+} entry SOCE). This SOCE protocol was used to provoke mitochondrial Ca^{2+} uptake. In "10 mM $CaCl_2$" panels: vertical dashed lines mark 0.3 μM [Ca^{2+}]c; at this [Ca^{2+}]c, [Ca^{2+}]m was higher in KO9 and KO28 *vs*. EV-Ctrl4. Further details are provided in the Methods section.

PiC-depleted HEK cells, and, for the same $[Ca^{2+}]c$, $[Ca^{2+}]m$ is higher in both clones of PiC-KO cells. The latter can be appreciated in the lower panels of Figure 7 (10 mM $CaCl_2$ condition) by comparing $[Ca^{2+}]m$ levels (y-axis) for $[Ca^{2+}]c$ (x-axis) ~ 3 µM (blue or orange vertical dashed lines). It should be noted that cells were treated with CGP37157 to inhibit Ca^{2+} efflux from mitochondria which would otherwise have complicated the interpretation of the data.

The results shown in Figure 7 are consistent with lesser buffering of Ca^{2+} in the mitochondrial matrix when PiC is depleted. The power of the above-described approach is in the ability to compare $[Ca^{2+}]m$ at the same $[Ca^{2+}]c$ among the genotypes (i.e., that higher or lower $[Ca^{2+}]c$ leads to higher or lower [Ca2+]m, respectively, would be a trivial result, thus it is important to compare $[Ca^{2+}]m$ at the same $[Ca^{2+}]c$). Going forward, it will be of interest to analyze the expression level of the components of the mtCU, since the ability of lower $[Ca^{2+}]c$ to stimulate a rise in $[Ca^{2+}]m$ in the PiC-KO clones exposed to the 2 mM

SOCE regime could indicate a lower expression of MICU1 leading to a lower threshold for mitochondrial Ca^{2+} uptake in PiC-depleted HEK cells as was observed for HeLa cells, mouse embryonic fibroblasts and mouse hepatocytes depleted of MICU1 (Csordas et al., 2013; Antony et al., 2016).

Because PiC depletion in mouse skeletal muscle mitochondria is an important model of PiC depletion in a complex biological system, we aimed to set up a protocol to measure [Ca2+]m simultaneous with [Ca2+] in the buffer ($[Ca^{2+}]b$) in which isolated mitochondria are suspended. We successful set up a protocol, as demonstrated in Figure 8 in which mitochondria in suspension had been pre-depleted of Pi and loaded with a Ca^{2+} sensor dye were then challenged with a pulse of 10 mM $CaCl_2$. Measurement of $[Ca^{2+}]b$ in mitochondria from Control (black and grey traces) and mice with PiC-depletion in skeletal muscle (red and light red traces) (mice bred and muscle harvested under another IACUC protocol) demonstrated robust, and similar, mitochondrial Ca^{2+} uptake as indicated by the clearance of $CaCl_2$ from the buffer ("Ca^{2+} Clearance" panel). That mitochondria were able to rapid and avidly take up Ca^{2+} demonstrates that the Pi pre-depletion and Ca^{2+} dye loading

did not adversely affect mitochondrial membrane potential. Future experiments will, however, also quantify membrane potential.

In Figure 8 (next page), simultaneous measurement of $[Ca^{2+}]m$ revealed a higher rise in $[Ca^{2+}]m$ in PiC-depleted mitochondria ("Mitochondrial Ca^{2+}"panel). Because mitochondrial Ca^{2+} uptake ("Ca^{2+} clearance") was similar in both genotypes, the elevated $[Ca^{2+}]m$ in PiC-depleted mitochondria indicates a lesser capacity to chelate Ca^{2+}, similar to what we observed in both clones of HEK-PiC-KO cells (Figure 7). In left and middle panels of Figure 8, grey and light red traces show signals after addition of Ruthenium Red (RuR) to inhibit mtCU; mitochondrial Ca^{2+}, or a rise in $[Ca^{2+}]m$, are no longer apparent after RuR addition. The right panel of Figure 8 shows the quantification for 2 technical replicates.

Accomplishments: In conclusion, we have successfully set up robust protocols to evaluate mitochondrial Ca^{2+} uptake and the levels of free mitochondrial matrix Ca^{2+}, in both HEK cells and isolated skeletal muscle mitochondria, allowing us to evaluate the impact of PiC depletion on matrix Ca^{2+} buffering. Our preliminary data from HEK cells and isolated skeletal muscle mitochondria both indicate that PiC depletion is accompanied by lesser Ca^{2+} buffering in the mitochondrial. While this result may not be thought of as surprising, since indirect evidence has suggested that Pi chelates a large amount of Ca^{2+} in the mitochondrial matrix, the higher free matrix Ca^{2+} in Ca^{2+} depleted cells or skeletal muscle mitochondria though clear was not substantial. This suggests that either an alternate Pi uptake pathway transport Pi into the matrix, as suggested by the bioenergetics studies described above, and/or that another buffering species is present in the matrix. What is unexpected is that the lesser buffering in HEK cells depleted of PiC occurs in the context of a lack of any bioenergetics defect in those cells. This suggests that, by an unknown mechanism, the PiC-depleted HEK cells prioritized the minimal Pi that was available for ATP synthesis, at least in the context of the studies conducted in permeabilized cells using maximal [ADP] and maximal [substrate].

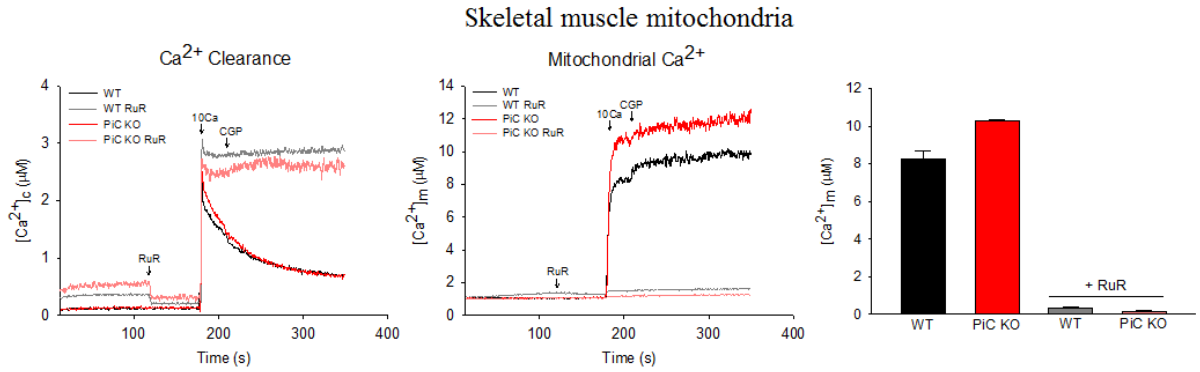


Figure 8. Ca^{2+} dynamics in isolated mouse skeletal muscle mitochondria in response to addition of 10 mM $CaCl_2$. Please note that skeletal muscle was harvested from mice bred and maintain under a separate IACUC protocol and that DOD funds were not used for the purchase, breeding or maintenance of mice. Mitochondria were pre-depleted of inorganic phosphate and loaded with the fluorescent Ca^{2+} sensor mtCepia. "Ca^{2+} clearance" refers to disappearance of Ca^{2+} from the buffer in which mitochondria were suspended and reflects Ca^{2+} uptake by mitochondria (thapsigagin was included to prevent Ca^{2+} uptake by any endoplasmic reticulum that remained in the preparation). CGP was added to prevent efflux of Ca^{2+} from mitochondria via the NCLX. "Mitochondrial Ca^{2+}" reflects the concentration of Ca^{2+} free in the mitochondrial matrix. RuR: ruthenium red was added to inhibit the mitochondrial Ca^{2+} uniporter, done as a control to investigate if this was the only Ca^{2+} uptake pathway; lack of changes with 10 mM when RuR was present indicates that Ca^{2+} uptake was only via the uniporter. Rightmost panel: averages from 2 technical replicates. These data show that mitochondrial Ca^{2+} uptake was similar for control and PiC-depleted skeletal muscle (KO) mitochondria (similar Ca^{2+} clearance traces) but that matrix free Ca^{2+} was higher in KO mitochondria; this reflect lesser buffering of Ca^{2+} in the matrix. Further details of methods are provided in the Methods section of the text.

Next steps: Going forward it will be of interest to adapt the SOCE protocol (Figure 7) to the Seahorse XF24 environment so that bioenergetics can be evaluated in the context of stimuli that elicit mitochondrial Ca^{2+} uptake and thus trigger matrix buffering of Ca^{2+}. We have previously successfully adapted a SOCE protocol in HeLa cells studied in the XF24 instrument (Csordas et al., 2013) thus we anticipate being able to set this up for the HEK model. Additionally, upcoming studies will focus on testing other HEK-PiC-KO clones as well as studying the Ca^{2+} uptake and buffering phenotypes in isolated skeletal muscle mitochondria from mice with skeletal muscle-specific depletion of PiC.

Section 4: Aim 3 Accomplishments

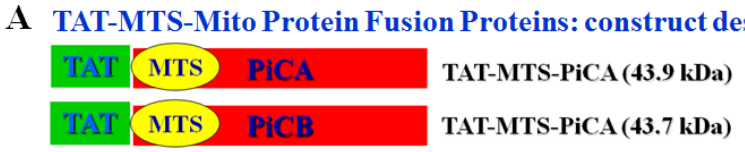
4.1. Rationale and Goal

The first goal of Aim 3 was to construct and test TAT-PiC as a means of rescuing deleterious phenotypes caused by PiC loss. Humans (and mice) have a documented alternatively spliced exon 3A and 3B, generating two PiC isoforms, A and B. Thus, we designed and constructed both TAT-PiC-A and TAT-PiC-B *human* isoforms. The natural mitochondrial targeting sequence (MTS) of PiC (49 aa) was used.

4.2. Approach, Methods and Results

The sequence encoding the chimeric protein was inserted into a pet28 expression vector under the control of the T7 promoter. Figure 9A demonstrates the schematic structure of the final chimeric proteins: TAT-MTS-PiCA and TAT-MTS-PiCB. The coding sequence of both chimeric proteins was confirmed by sequencing analysis. Full amino acid sequences of TAT-MTS-PiCA and TAT-MTS-PiCB fusion protein are shown in Figure 9B.

At first, we performed experiments with the TAT-MTS-PiCA fusion protein. The TAT-MTS-PiCA chimeric protein was expressed in various bacterial expression systems and under different conditions (not shown). The most successful expression system proved to



B Mitochondrial PiC isoforms A & B:

	TAT-MTS-PiCA	TAT-MTS-PiCB
DNA seq. Length	1188	1185
Amino acids	MGSSHHHHHHSSGLVPRGSHMRKKRRQRRRGSGSFSSVAHLARANPFNTPHL QLVHDGLGDLRSSSPGPTGQPRRPRNLAAAAVEEQYSCDYGSGRFFILCGLGG IISCGTTHTALVPLDLVKCRMQVDPQKYKGIFNGFSVTLKEDGVRGLAKGWA PTFLGYSMQGLCKFGFYEVFKVLYSNMLGEENTYLWRTSLYLAASASAEFFA DIALAPMEAAKVRIQTQPGYANTLRDAAPKMYKEEGLKAFYKGVAPLWMRQ IPYTMMKFACFERTVEALYKFVVPKPRSECSKPEQLVVTFVAGYIAGVFCAIV SHPADSVVSVLNKEKGSSASLVLKRLGFKGVWKGLFARIIMIGTLTALQWFIY DSVKVYFRLPRPPPPEMPESLKKKLGLTQ	MGSSHHHHHHSSGLVPRGSHMRKKRRQRRRGSGSFSSVAHLARANPFNTPHL QLVHDGLGDLRSSSPGPTGQPRRPRNLAAAAVEEYSCEFGSAKYYALCGFGG VLSCGLTHTAVVPLDLVKCRMQVDPQKYKGIFNGFSVTLKEDGVRGLAKGW APTFLGYSMQGLCKFGFYEVFKVLYSNMLGEENTYLWRTSLYLAASASAEFF ADIALAPMEAAKVRIQTQPGYANTLRDAAPKMYKEEGLKAFYKGVAPLWMR QIPYTMMKFACFERTVEALYKFVVPKPRSECSKPEQLVVTFVAGYIAGVFCAI VSHPADSVVSVLNKEKGSSASLVLKRLGFKGVWKGLFARIIMIGTLTALQWFI YDSVKVYFRLPRPPPPEMPESLKKKLGLTQ
	first methionine His tag TAT mts PiCVar1	first methionine His tag TAT mts PiCVar2

Figure 9. TAT-PiC fusion protein design for isoforms A and B of PiC. Panel A presents a schematic of the construct design that includes a mitochondrial targeting sequence (MTS). Panel B shows the actual sequences for both constructs.

be expression in the HMS bacterial strain, growth at 37°C following induction with 1mM IPTG for 2-3 hrs at 37°C.

Following expression, various subcellular fractions were prepared: whole cell extract (WCE), soluble fraction (Sol) and inclusion bodies (IB), separated on SDS-PAGE, and characterized both by Coomassie blue staining and by immunoblot analyses, using anti-His and human anti-PiC antibodies (Sigma). Coomassie blue staining revealed bands with estimated masses corresponding to those of the over-expressed protein: ~43kDa. Western blot performed with antibodies specific to His (Figure 10) and human PiC (not shown) confirmed the identity of the fusion protein. As expected, being an inner membrane mitochondrial protein, when analyzed by immunoblot, the subcellular fraction that held a

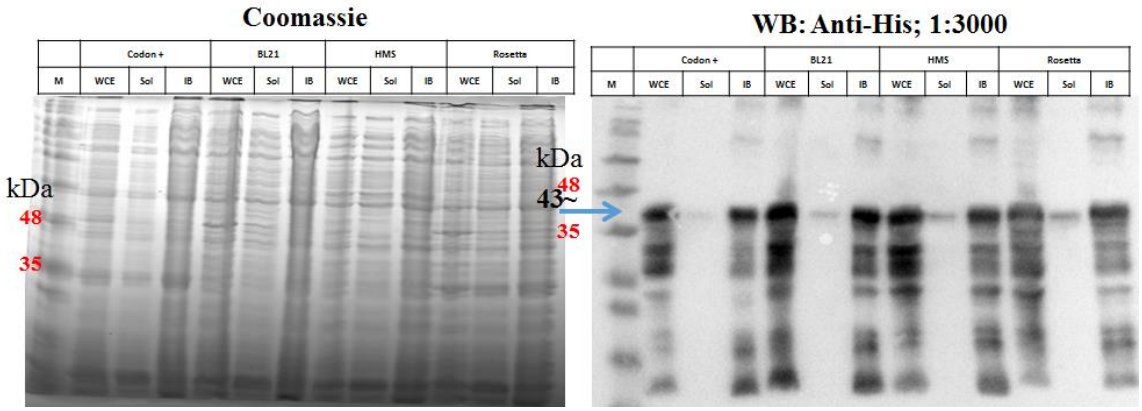


Figure 10. TAT-PiCA is found in inclusion bodies. Subcellular fractions (WCE: whole cell extract; Sol: soluble fraction; IB: inclusion bodies) were electrophoresed and probed with Anti-His antibodies (right panel). The major band was found at ~43 kDa which is the calculated molecular weight for the His-tagged TAT-PiCA protein. Coomassie blue-stained gel (left panel) shows electrophoresed protein in all fractions.

larger portion of the TAT-MTS-PiCA fusion protein was the IB in the bacteria (not shown). These analyses confirm the successful expression of the in-frame, full-length fusion protein.

A frozen pate of the bacteria were thawed and suspended in lysis buffer (20 mM Tris-HCl, pH 8.0; 0.05 M NaCl; 0.2 mg/ml lysozyme; 1 mM phenylsullfonyl fluoride (PMSF)) for 30 minutes at room temperature, followed by sonication (the last calibration is using a

microfluidezer to disrupt the bacterial cells instead of using a sonicator). A sample was taken from the lysed cells and marked as whole cell extract (WCE). The rest of the cells were then centrifuged at 17,500 g for 30 min. The supernatant (marked as soluble fraction; sol) was removed and the pellet (containing inclusion bodies) was suspended in denaturation buffer (6 M urea; 20 mM Tris-HCl, pH 8.8; 0.05 M NaCl; 5mM β mercaptoethanol) and continuously stirred for 90 minutes. The solution was cleared by centrifugation at 17,500 g for 30 min. The supernatant, containing denatured proteins, was collected and marked as inclusion bodies (IB).

Accomplishment: The denatured chimeric proteins in 6M urea were filtrated and a final concentration of 10 mM imidazole was added. The protein was subjected to immobilized metal affinity chromatography using 5 ml His-Trap columns, and was eluted with a linear imidazole gradient of 10–500 mm (Figure 11, rightmost panel). Again, being a membrane protein, calibration experiments were performed to allow binding of the fusion protein to the affinity column (results not shown). Figures 11 shows the affinity purification of the TAT-MTS-PiCA fusion protein.

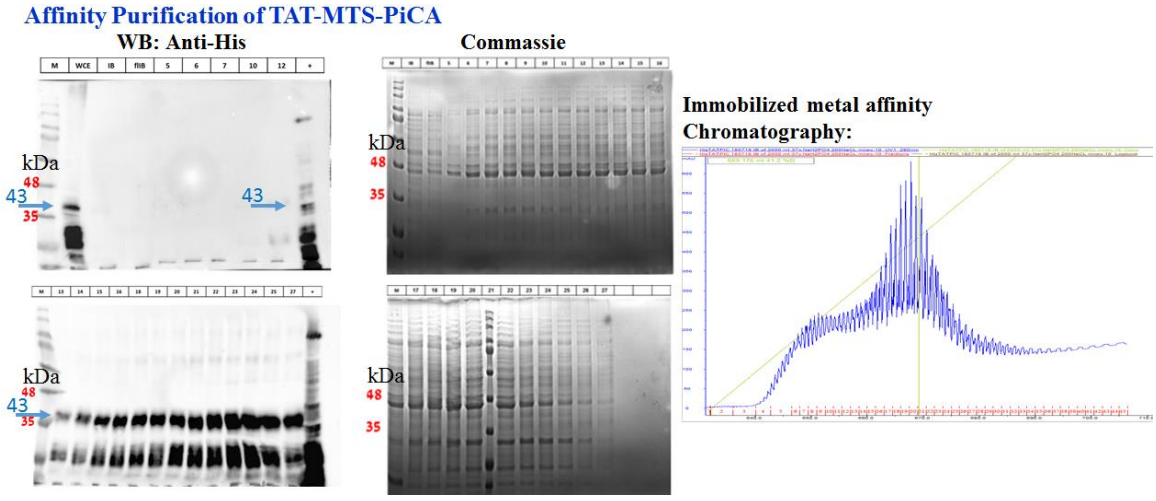


Figure 11. Affinity chromatography to TAT-PiCA. Left upper and lower panels show immunoblot of elution fractions, probed with an anti-His antibody. Labels along the top indicate whole cell lysate (WCE) followed by the numbers of the collected fractions. TAT-PiCA runs at ~43 kDa and appeared most abundantly in fractions 16 through 27. Gels on the right show correctly electrophoresed protein in all fraction, demonstrated using Coomassie blue staining. The rightmost panel displays the chromatogram readout.

Next steps: We are now in the process of trying to further purify the fusion protein by using other columns such as ion-exchange column as well as finding conditions for storage of the fusion protein in an active form for biological experiments in cell culture. The cell culture experiments will determine if TAT-PiCA can rescue phenotypes induced by PiC depletion. At present we are fairly confident that the delayed cell proliferation in HEK-PiC-KO clones K06 and K09 can be used as a straightforward phenotype on which to determine the ability of TAT-PiCA to rescue phenotypes. If more than one HEK-PiC-KO clone displays a robust bioenergetics phenotype when the cells are studied in the intact state, then this phenotype can also be used to evaluate TAT-PiCA rescue. Alternatively, we will evaluate the ability of TAT-PiCA to rescue the bioenergetics phenotype induced by acute major PiC depletion in HeLa cells (Seifert et al., 2016).

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4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

-This project will allow us to understand whether the mitochondrial phosphate carrier (PiC) is absolutely required for the transport of inorganic phosphate into the mitochondrial matrix. Although the latter has in fact been assumed, it was never tested directly because of the lack of adequate models. This project will utilize several new models of PiC depletion to specifically test, for the first time, the requirement of PiC for oxidative phosphorylation (Aim 1) and buffering of Ca^{2+} that enters the mitochondrial matrix (Aim 2). This project will also evaluate how skeletal muscle adapts to PiC which is particularly relevant in the context of mutations in the human gene that encodes PiC that result in myopathies (Aims 1 and 2).

-This project will generate TAT fusion proteins for both PiC isoform (A and B). These may have therapeutic potential for individuals suffering from myopathy caused by variants of the human gene encoding PiC.

- **What was the impact on other disciplines?**

-Development of the HEK-PiC-KO model can be useful in other disciplines, namely for those with interests in the detailed study of mitochondrial inner membrane transporters.

-These studies allow us to evaluate (mal)adaptive mechanisms that arise from mitochondrial dysfunction (due, here, to PiC depletion) and thus to gain broader understanding of cellular and tissue responses to mitochondrial dysfunction. The utility of the latter is 1) to gain a better understanding of basic adaptive mechanisms, and 2) to gain better insight into the pathogenesis of mitochondrial disease which, as is becoming increasingly apparent, is not merely induced by a decline in oxidative phosphorylation.

-Development of TAT-PiC fusion proteins is particularly challenging because PiC is a membrane protein. Development of methods to purify and store TAT-PiCA in an active conformation might prove useful for the development of other TAT fusion proteins made for membrane proteins.

- **What was the impact on technology transfer?**

  NOTHING TO REPORT

- **What was the impact on society beyond science and technology?**

-This project has provided numerous training opportunities for Masters and Doctoral students as well as post-doctoral fellows, in terms of learning new techniques as well as learning about mitochondrial bioenergetics, cellular Ca^{2+} handling and mitochondrial disease.

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

In addition to using the primary muscle cells originally proposed, we developed a model of CRISPR-induced knockout of PiC in HEK 293T cells. The reason for this addition is that, even though development of a CRISPR model is laborious and requires testing of multiple clones, HEK cells are straightforward to culture and can serve as a workhorse model to test hypotheses especially those in Aim 2 (mitochondrial Ca^{2+} handling) which address fundamental questions that are less tied to a cell or tissue type. We reasoned that another benefit of the practicality of the HEK cell model is its use to evaluate the ability of the TAT-PiC fusion proteins to rescue phenotypes induced by PiC loss.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

The mouse work was delayed for the following reason which has also been explained to the Program Officer, Kathryn Argue. We had substantial breeding problems in the mouse colony that was to provide us with adult-onset skeletal muscle-specific PiC depletion. These mice are generated from mice expressing a transgene that drives Cre recombinase expression only in skeletal muscle upon tamoxifen injection (HSA-MCM: Human Skeletal Actin-MerCreMer) and mice that express floxed PiC alleles ($PiC^{fl/fl}$). We finally opted to introduce new HSA-MCM mice which were recently purchased from Jackson Laboratories (under a separate IACUC protocol and not using DOD funds). We hope that the introduction of these new mice will result in a stable colony; this colony should be available in ~8 months. We have also succeeded in generating mice with *constitutive* PiC depletion in skeletal muscle by breeding $PiC^{fl/fl}$ mice with HSA-Cre (tamoxifen not needed for Cre recombinase expression). These mice were bred under another IACUC protocol and not using DOD funds. This colony appears to be stable and we expect to have sufficient numbers of experimental mice within a couple of months.

- **Changes that had a significant impact on expenditures**
  No changes/Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  Nothing to Report

- **Significant changes in use or care of human subjects**
  Nothing to Report

- **Significant changes in use or care of vertebrate animals.**
  Nothing to Report

- **Significant changes in use of biohazards and/or select agents**
  Nothing to Report

6. PRODUCTS, INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

- **Publications, conference papers, and presentations**
    - **Journal publications.**

      No. Nothing to Report
    - **Books or other non-periodical, one-time publications.**

      No. Nothing to Report
    - **Other publications, conference papers, and presentations.**

      No. Nothing to Report
    - **Website(s) or other Internet site(s)**

      No. Nothing to Report
    - **Technologies or techniques**

      Nothing to Report
    - **Inventions, patent applications, and/or licenses**

      Nothing to Report
    - **Other Products**

      Regarding “Other products”, we have products in progress, namely the TAT-PiCA fusion protein and the HEK-PiC-KO model. However, both these are in the testing and development phase, respectively. Therefore, it does not seem appropriate to report them at this time. Therefore, the answer is: NOTHING TO REPORT.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Erin Seifert
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Dr. Seifert is the Principal Investigator of this project (Overall decisions about models and studies to be conducted, communication with subawardees and co-investigators, design of Aim 1 studies, direct supervision of Lanren Anderson-Pullinger and Aish Sivaramakrishnan, contributions to design of Aim 2 studies, coordination of testing of TAT-PiCA protein in cell culture models and design of those studies, responsible for progress reports and quality control of all data and scientific integrity of all studies)
Funding Support:	NA

Name:	Gyorgy Hajnoczky
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Gyorgy Hajnoczky has performed work in the area of Aim 2 (design of the Ca2+ studies and supervision of Valentina Debattisti)
Funding Support:	NA

Name:	Lauren Anderson-Pullinger
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA

Nearest person month worked:	1
Contribution to Project:	Lauren Anderson-Pullinger has performed work in the area of Aims 1 and 2 (immunoblot analysis of the HEK cell model)
Funding Support:	NA

Name:	Valentina Debattisti
Project Role:	Post-Doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	4
Contribution to Project:	Valentina Debattisti has performed work in the area of Aim 2 (measurements of Ca2+, cell culture for the HEK model)
Funding Support:	NA

Name:	Aishwarya Sivaramakrishnan
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	9
Contribution to Project:	Aishwarya Sivaramakrishnan has performed work in the area of Aim 1 (qPCR, immunoblot analysis of HEK cells)
Funding Support:	NA

Name:	Samar Zabat
Project Role:	Technician (Hebrew University/Consortium)
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	6

Contribution to Project:	Samar Zabat performed all the research work. Designed, construct and performed expression of the new TAT-MTS-PiC fusion proteins. Also in the process of purification of the fusion protein, and its biochemical characterization.
Funding Support:	NA

Name:	Tejvir S. Khurana
Project Role:	PI (Univ of Pennsylvania/Consortium)
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1
Contribution to Project:	Dr. Khurana has performed the work in the area of planning the experiments that will help analyze the role of mitochondrial phosphate carrier depletion in skeletal muscle by undertaking physiological assessment of mice (control and mutant) provided by Dr. Seifert at various time points using physiological apparatuses (commercial and custom built) available in our lab. He has been involved with planning and helping optimize the use of various physiological apparatus in the lab including force measurements and breathing analyses based mice that are currently available in the lab.
Funding Support:	NA

Name:	Emanuele Loro
Project Role:	Res Assoc (Univ of Pennsylvania/Consortium)
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	Dr. Loro has performed the work in the area of planning experiments, optimizing equipment and

	making measurements that will help analyze the role of mitochondrial phosphate carrier depletion in skeletal muscle by undertaking physiological assessment of mice (control and mutant) provided by Dr. Seifert at various time points using physiological apparatuses (commercial and custom built) available in our lab. He has been involved with planning and helping optimize the use of various physiological apparatus in the lab including force measurements and breathing analyses using mice that are currently available in the lab..
Funding Support:	NA

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Seifert, Erin
Current Research Support:

The Below Research support/award ended 12/31/17:
Title: Regulation of Lipid and Glucose Metabolism by ACYL-COA Thioesterase2
Role: Principle Investigator
Effort: 0 CM
Agency: American Heart Association
Grants Officer/Address: Grants Officer Unknown, 7272 Greenville Avenue, Dallas, TX 75231
Performance Period: 1/1/2013-**12/31/2017** - currently in No Cost Extension
Level of Funding: $70,000
Goal: The major goal of this project is to define the biological role and regulation of acyl-CoA thioesterase-2 in the heart, primarily using an overexpression model. This project also explores the regulation of acyl-CoA thioesterase-2 enzymatic activity.
Specific Aims: Aim 1: What is the role of Acot2 in lipid handling the liver? Acot2 will be overexpressed in the liver of mice and in primary liver cells in culture. Aim 2: What is the role of Acot2 in lipid handling in the heart? Acot2 will be overexpressed in primary heart cells in culture. In both Aims 1 and 2, mitochondrial and cystosolic lipid handling will be evaluated, as well as metabolic flexibility and insulin signaling. Aim 3: Do the characteristics of the Acot2 enzyme endow Acot2 with a unique function among the mitochondrial Acots?
Overlap: No Overlap

The Below Research support/award ended 07/01/17:
Title: Pathogenesis of myopathies caused by mitochondrial phosphate carrier mutations
Role: Principle Investigator
Effort: 0 CM
Agency: United Mitochondrial Disease Foundation
Grants Officer/Address: Phil Yeske, Ph.D.
Performance Period: 07/01/2014-**07/01/2017**
Level of Funding: $60,000
Goal: This grant focuses evaluating novel PiC mutations in patient cells, and analyzing the cellular metabolic consequences of PiC deficiency using cell lines.
Specific Aims: Aim 1: To identify alternate mechanisms of mitochondrial Pi uptake. This will be done using patient cells as well as cell lines in which PiC-B (the PiC isoform expressed in these cells) has been knocked down. Aim 2: To test whether PiC deficiency is associated with dysregulation of cytoplasmic and mitochondrial Ca^{2+}, and Ca^{2+} regulated functions, using high affinity compartment-specific Ca^{2+} probes in patient cells and cell lines. Aim 3: To analyze the cellular metabolic consequences of PiC deficiency, using patient cells as well as cell lines in which PiC-B has been knocked down. State-of the-art bioenergetics and metabolomics analyses will be used.
Overlap: No Overlap

Title: Molecular Mechanisms of Mitochondrial Ca2+ Transport
Identifier: R01 GM102724
Role: Co-Investigator
Effort: 1.92 CM
Agency: NIH/NIGMS
Grants Officer/Address: Eileen Hyde, Hydee@nigms.nih.gov
Performance Period: 02/01/15-02/28/2021
Level of Funding: $228,000
Goal/Specific Aims: 1) to test a mechanistic model for the Ca2+-dependent control of mtCU based on MICU1's interactions with MCU and EMRE and dimerization with MICU1/2/3; 2) to determine the functional and pharmacological relevance of the tissue-specific differences in the molecular composition of the mtCU; 3) to test the role of mitochondrial calcium signaling in mitochondrial metabolism and stress responses of the liver; 4) to determine the MICU1-dependence of neuronal calcium signaling and function.
There is no overlap with the current application.

The Below Research support/award end date and CM has been updated:
Title: Impact of Frataxin deficiency on cardiac substrate metabolism
Role: Principle Investigator
Effort: 4.08 CM
Agency: Friedreich Ataxia Research Alliance (FARA)
Grants Officer/Address: Jennifer Farmer
Performance Period: 09/01/2016-**12/31/18**
Level of Funding: $150,000
Goal: To determine the mechanisms of pathogenesis in the heart caused by loss of Frataxin
Specific Aims: Specific Aims: Aim 1: we hypothesize that lipid overload will develop in cardiac mitochondria, with a subsequent accumulation of acyl-CoA and acetyl-CoA species, a lesser ability to switch to glucose oxidation, and hyperacetylation of mitochondrial proteins. Aim 2: Skeletal muscle, like the heart, relies on mitochondrial ATP synthesis. Thus it is hypothesized that skeletal muscle deficient in Fxn will also exhibit metabolic abnormalities, and that these will correlate with the progression of cardiac dysfunction.
Overlap: No Overlap

The Below Research support/award was reported as pending and was funded:
Title: Regulation of substrate metabolism in skeletal muscle by mitochondrial thioesterases
Identifier: R01 DK109100
Role: Principal Investigator
Time Commitment: 4.8 Calendar Months
Supporting Agency: NIH
Contracting/Grants Officer: Craig Bagdon, bagdonc@niddk.nih.gov
Performance Period: 7/01/17-6/30/22
Level of Funding: $250,000
Project Goals/Specific Aims: The central goal is to determine the biological role and mode of action of the mitochondrial enzyme, Acot2.
There is no overlap with the current application.

The Below Research support/award was reported as pending and was funded:

Title: Pathogenesis of Myopathies Caused by Novel Mitochondrial Phosphate Carrier Mutations
Role: Principle Investigator
Effort: 5.4 CM
Agency: NIH
Grants Officer/Address: Vernon Anderson, Ph.D.
Performance Period: 07/01/2017-06/30/2021
Level of Funding: $192,000
Goal: To investigate the fundamental role of the PiC in mitochondrial Pi transport, the importance of PiC-mediated Pi transport for oxphos and mitochondrial Ca^{2+} uptake and buffering, as well as the pathogenesis of PiC deficiency in skeletal muscle. These studies will also allow us to delineate the (mal)adaptive mechanisms due to severe mitochondrial dysfunction, which are poorly understood for mutations in nuclear DNA-encoded mitochondrial proteins.
Specific Aims: Aim 1: To test for the role of PiC in skeletal muscle and for critical mechanisms counteracting PiC deficiency. Aim 2: To test whether PiC deficiency causes dysregulation of cytoplasmic and mitochondrial Ca2+, and Ca2+ regulated functions.
Overlap: Currently, Aims1 and 2 of the present DoD application overlap 100% with this R01 application. The NIH NIGMS Program Officer, Vernon Anderson, is aware of this overlap and is willing to revise the Aims of the NIH grant such that there is no longer overlap with Aim 2 of the DoD grant. Additionally, Dr. Anderson has agreed to remove part of Aim 1 of the NIH such that part of Aim 1 of the DoD becomes unique.
Aim 3 of the DoD application has no overlap with the R01 application.

Hajnoczky, Gyorgy
Current Research Support

The Below Research support/award end date and CM has been updated:
Title: Mitochondrial Calcium Signaling in Cell Death
Identifier: R01 GM059419
Hajnoczky PI
Time Commitment: **.12** Calendar Months
Supporting Agency: NIH/NIGMS
Contracting/Grants Officer: Kimberly Cornwell, cornwek@nigms.nih.gov
Performance Period: 03/01/00–**2/28/2019** (NCE)
Level of Funding: $211,150
Project Goals/Specific Aims: This award supports the PI's program to address the VDAC2-dependent mechanisms of mitochondrial apoptosis and to test the hypothesis that that local regulation by mitochondrial ROS supports both Bid-and IP3R-mediated cell killing.
Overlap: None

The Below Research support/award end date and CM has been updated:
Title: Mechanisms of Pulsatile Calcium Signaling
Identifier: R01 DK051526
Hajnoczky PI
Time Commitment: **.12 Calendar Months**
Supporting Agency: NIH/NIDDK
Contracting/Grants Officer: Natasha Loveless, lovelessnd@mail.nih.gov
Performance Period: 4/19/2012 – **3/31/2019** (NCE)
Level of Funding: $217,500
Project Goals/Specific Aims: The major goals of this project are to define the mechanisms underlying generation of cytosolic calcium spikes and oscillations in liver and other cell types. The hypothesis being evaluated is that a local coupling between endoplasmic reticulum and mitochondria permits highly efficient propagation of calcium spikes to the mitochondrial matrix, which in turn, effectively controls mitochondrial dynamics and function. Aim#2 of this application was focused on the role of MICU1 in mitochondrial calcium uptake. The vast majority of the proposed studies has been completed and were published in Csordas et al 2013 Cell Metabolism. The remaining efforts focus on completion of Aims#1 (dependence of calcium delivery to mitochondria on structure of the IP3 receptor) and #3 (calcium-dependent control of mitochondrial dynamics).
Overlap: None

The Below Research support/award ended 7/31/18:
Title: Cell Death in Alcoholic Heart and Muscle
Identifier: U01 AA021122
MPI: Hajnoczky & Rubin
Time Commitment: 0 Calendar Months
Supporting Agency: NIH/NIAAA

Contracting/Grants Officer: Deborah Hendry, dhendry@mail.nih.gov
Performance Period: 5/1/2012 – **7/31/18**
Level of Funding: $225,000
Project Goals/Specific Aims: The major goals are to (1) determine the mechanisms by which chronic EtOH consumption affects cellular stress responses in the heart and promotes cardiac cell death and tissue damage in humans, and (2) study the effects of chronic EtOH consumption on apoptosis in skeletal muscle in humans and animals.
Overlap: None

The Below Research support/award CM and level of funding has been updated:
Title: Molecular Mechanisms of Mitochondrial Ca2+ Transport
Identifier: R01 GM102724
Hajnoczky PI
Time Commitment: **1.44 Calendar Months**
Supporting Agency: NIH/NIGMS
Contracting/Grants Officer: Eileen Hyde, Hydee@nigms.nih.gov
Performance Period: 2/1/2015 – 02/28/21
Level of Funding: **$205,200**
Goal/Specific Aims: 1) to test a mechanistic model for the Ca2+-dependent control of mtCU based on MICU1's interactions with MCU and EMRE and dimerization with MICU1/2/3; 2) to determine the functional and pharmacological relevance of the tissue-specific differences in the molecular composition of the mtCU; 3) to test the role of mitochondrial calcium signaling in mitochondrial metabolism and stress responses of the liver; 4) to determine the MICU1-dependence of neuronal calcium signaling and function.
There is no overlap with the current application.

Title: Redox regulation of intracellular calcium signaling
Identifier: R01 DK103558
MPI: Hajnoczky & Joseph
Time Commitment: .96 Calendar Months
Supporting Agency: NIH/NIDDK
Contracting/Grants Officer: Craig Bagdon, bagdonc@niddk.nih.gov
Performance Period: 4/01/2015 – 3/31/19
Level of Funding: $202,500
Project Goals/Specific Aims: The long-term goal of the proposal is to obtain a detailed understanding of how oxidative stress impacts intracellular Ca2+ signaling under normal and disease conditions.
No scientific or budgetary overlap with current application.
Overlap: None

The Below Research support/award CM, performance period, and level of funding has been updated:
Title: Study of the mitochondrial-cellular response to environmental stress by flourescence imaging
Identifier: R21 ES025672/4R33 ES025672
MPI: Hajnoczky & Csordas

Time Commitment: **2.16 Calendar Months**
Supporting Agency: NIH/NIEHS
Contracting/Grants Officer: Barbara Gittleman, gittlemanbj@niehs.nih.gov
Performance Period: **7/14/17-06/30/20* (R33)**
Level of Funding: **$336,084**
Project Goals/Specific Aims: This proposal focuses firstly on developing a new, genetically-targeted toolkit to perturb and measure the reactive oxygen species (ROS) and calcium signals in a sensitive and specific manner with a resolution power at the level of specific subcompartments of the mitochondria. Secondly, the novel toolkit will be applied to mice to enable study the effect of various environmental agents on ROS and calcium signals in situ in the liver, heart and skeletal muscle. Finally (R33) the project will study the specific involvement of ROS and calcium in the stress pathways triggered by arsenic, cadmium and dioxin and will specifically test the novel hypothesis that environmental stress induced by these agents causes impaired mitochondria-endoplasmic/sarcoplasmic reticulum (ER/SR) functional and structural coupling, providing an important mechanism underlying cell injury in various tissues, including the liver, cardiac and skeletal muscle.
Overlap: None

The Below Research support/award ended 6/30/18:
Title: Altered mitochondria-ER signaling as a cause of chemotherapy resistance
Identifier: R21 CA198430-01
Hajnoczky PI
Time Commitment: 0 Calendar Months
Supporting Agency: NIH/Through CHOP sub award
Contracting/Grants Officer: Candace M. Cofie, Candace.cofie@nih.gov
Performance Period: 7/1/16-**6/30/18**
Level of Funding: $135,259
Project Goals/Specific Aims: will be to evaluate the ER-mitochondrial (1) physical and (2) calcium coupling in therapy sensitive and resistant neuroblastoma using electron microscopy and fluorescence based calcium imaging.
Overlap: None

The Below Research support/award reported as pending now funded:
Title: Pathogenesis of Myopathies Caused by Novel Mitochondrial Phosphate Carrier Mutations
Identifier: R01 GM123771-01A1
Role: Co- Investigator
Effort: .24 CM
Agency: NIH
Grants Officer/Address: Vernon Anderson, Ph.D.
Performance Period: 09/01/17-08/31/21
Level of Funding: $192,500
Goal: To investigate the fundamental role of the PiC in mitochondrial Pi transport, the importance of PiC-mediated Pi transport for oxphos and mitochondrial Ca^{2+} uptake and buffering, as well as the pathogenesis of PiC deficiency in skeletal muscle. These studies will also allow us to delineate the (mal) adaptive mechanisms due to severe mitochondrial dysfunction, which are poorly understood for mutations in nuclear DNA-encoded mitochondrial

proteins.
Specific Aims: Aim 1: To test for the role of PiC in skeletal muscle and for critical mechanisms counteracting PiC deficiency. Aim 2: To test whether PiC deficiency causes dysregulation of cytoplasmic and mitochondrial Ca2+, and Ca2+ regulated functions.Overlap: Currently, Aims1 and 2 of the present DoD application overlap 100% with this R01 application. The NIH NIGMS Program Officer, Vernon Anderson, is aware of this overlap and is willing to revise the Aims of the NIH grant such that there is no longer overlap with Aim 2 of the DoD grant. Additionally, Dr. Anderson has agreed to remove part of Aim 1 of the NIH such that part of Aim 1 of the DoD becomes unique.
Aim 3 of the DoD application has no overlap with the R01 application.

The Below Research support/award is new funding:
Title: Molecular composition of the mitochondrial calcium uniporter and cardiac pathophysiology
Identifier: R01 HL142271
Hajnoczky & Elrod -MPI
Time Commitment: 1.92 Calendar Months
Supporting Agency: NIH/NHLBI
Contracting/Grants Officer: Hubert Walters, waltersh@mail.nih.gov
Performance Period: 05/01/18-02/28/22
Level of Funding: $358,079
Project Goals/Specific Aims: This project tests the hypothesis, that adaptations to exercise and pathogenic stress in the heart are associated with and determined by plasticity in the mtCU molecular composition. Experiments will use novel genetic mouse models to modify mitochondrial Ca2+ uniporter composition, cardiac stressors to model physiologically and pathologically elevated workload, the assessment of cardiac hypertrophy and (dys)function, and live imaging techniques to reveal the pathophysiological importance of Ca2+ uniporter composition and thus to point to possible new therapeutic targets.
Overlap: None

The Below Research support/award is new funding:
Title: (PQ5) Relevance of VDAC2 heterogeneity for hepatic tumor growth and targeting
Identifier: R01 CA216254
Hajnoczky
Time Commitment: 1.56 Calendar Months
Supporting Agency: NIH/NCI
Contracting/Grants Officer: Candace M. Cofie, Candace.cofie@nih.gov
Performance Period: 05/22/18-04/30/23
Level of Funding: $265,331
Project Goals/Specific Aims: We postulate that the heterogeneity in VDAC2 and/or Bak abundance in the liver are important for hepatoma/ hepatocarcinoma (1) growth and (2) targeting by the combination of Mcl-1 inhibitor drugs and a cell permeable hydrocarbon stapled Bid BH3 peptide. This study will provide clues to the contribution of mitochondrial heterogeneity to hepatic tumorigenesis and test a novel tumor-selective targeting approach.
Overlap: None

Khurana, Tejvir S. (Consortium- Univ of Pennsylvania)
Current Research Support

The Below Research support/award ended 08/31/18:
Title: Pharmacotherapy to CounterACT parathion-induced NMJ dysfunction.
Role: Co-Principal Investigator
Effort: 0 CM
Agency: NIH/NINDS thru University of Massachusetts
Grants Officer: JETT, DAVID A
Grants Officer Address: Program Director, NIH CounterACT Research
National Institutes of Health, NINDS
6001 Executive Blvd.
NSC, Room 2177, MSC 9527
Bethesda, MD 20892-9527
Performance Period: 09/01/2013-**08/31/2018**
Level of Funding: $125,000
Goal: This project seeks to develop a therapy for parathion poisoning in murine animal models. These studies will evaluate the ability of pancuronium to protect the neuromuscular junction (NMJ) of murine animals following exposure to parathion.
Specific Aims:
Aim 1: To develop a novel rat model of the Intermediate Syndrome (IMS) after poisoning with parathion.
Aim 2: To characterize the *in vivo* muscle function and NMJ structural effects in this new rat IMS model during the acute poisoning and the recovery period.
Aim 3: To determine the effect of pancuronium therapy on *in vivo* muscle function in this new rat IMS model during the acute poisoning and the recovery period.
Aim 4: To determine the effect of pancuronium therapy on NMJ structure in this new rat IMS model of parathion poisoning during the acute poisoning and the recovery period.
Overlap: Nil

Title: Safety and Efficacy of Systemic Gene Therapy In Informative Models of DMD.
Role: Co-Principal Investigator
Effort: 0.1 CM
Agency: NIH
Grants Officer: Grants Officer: Nuckolls, Glen
Grants Officer Address: NIH/NINDS
Neuroscience Center, Room 2114
6001 Executive Blvd MSC Bethesda, MD 20892
Performance Period: 09/01/2015-08/31/2020
Level of Funding: $578,786
Goal: This project seeks to develop mini-utrophin gene therapy in murine and canine models of DMD.
Specific Aims:
Aim 1: We will characterize the maximal extent of phenotypic amelioration achievable following early neonatal administration of humanized AAV9mU to dystrophic mice, to test the hypothesis that durable, *complete* elimination of histological signs of muscular dystrophy will correlate with

normalization of all functional measurements relevant to the clinical course of DMD. Functional assays will include *in vivo* studies of *locomotive, cardiac, and respiratory function*, as well as complementary isolated organ studies to precisely quantify functional reserve.
Aim 2: We will perform detailed studies of the immune response to AAV9mUtrophin and AAV9mDystrophin in the deletional-null GSHPMD dog, to test the hypothesis that central tolerance will prevent recognition of mU-derived peptides, while mDystrophin-derived peptides will drive a dose-dependent, cytotoxic T-lymphocyte response associated with clinically severe myositis. *Myositis may be delayed but not prevented by transient immunosuppression because peripheral tolerance will not be established to the transgene product microdystrophin.* Assays will include quantification of peripheral T cells recognizing specific utrophin- , dystrophin-, and capsid-derived polypeptides.
Aim 3: We will establish dose-response relationships between AAV9mUtrophin dose and both the systemic recovery of the dystrophin-associated protein complex (DAPC) and the reversal of histological signs of myodegeneration in dystrophic dogs, to test the hypothesis that maintaining DAPC expression at or above wild type will completely reverse myopathology. Puppies will be injected with AAV at doses up to 3x10E15 at 1 kg, and will be followed until they achieve a body weight of 10 kg. In the absence of immunotoxicity, the systemically delivered AAV will protect muscle fibers from myonecrosis throughout this period of growth, in particular the MYH16(+) fibers that are the most sensitive indicators of disease in dogs.
Overlap: Nil

Title: IL15RA suppression: a novel therapeutic approach for metabolic disorders associated with circadian dysregulation.
Role: Principal Investigator
Effort: 0.1 CM
Agency: ITMAT (Pilot Grant)
Grants Officer: Lorri A. Schieri, MBA
Grants Officer Address: Director, Administration and Finance
Department of Systems Pharmacology and Translational Therapeutics
Institute for Translational Medicine and Therapeutics
10-123 Smilow Center for Translational Research
3400 Civic Center Boulevard, Building 421
Philadelphia, PA 19104-5158
Performance Period: 02/01/2016-1/31/2018 : Currently under No Cost Extension till 02-10/2019
Level of Funding: $150,000
Goal: This project seeks to seeks to identify ways in which the body's natural response to low oxygen can be most beneficially activated.
Specific Aims: Aim 1, we will determine the contribution of the CNS and/or muscle to the rhythmic nature of IL15Ra signaling.
Aim 2, we will test the ability of inhibiting IL15Ra signaling for improving the phenotype of metabolic disorders associated with circadian dysfunctions.
Overlap: None

Title: Utrophin High Altitude Adaptation: A Model for Chronic Hypoxia.
Role: Co-Principal Investigator
Effort: 1.2 CM

Agency: NIH
Grants Officer: LAPOSKY, AARON D.
Grants Officer Address: National Heart, Lung and Blood Institute
Two Rockledge Center, Suite 10042
6701 Rockledge Dr. MSC 7952, Bethesda, Maryland 20892-7952
Performance Period: 03/01/2016-2/28/2019
Level of Funding: $326,305
Goal: This project seeks to seeks to identify ways in which the body's natural response to low oxygen can be most beneficially activated
Specific Aims: SPECIFIC AIM 1 (R21 Phase): Identify the functionally important genetic variant of the *PHD2* gene that is associated with Tibetan adaptation to high altitude by using a combination of in vitro reporter gene assays, coimmunoprecipitation studies, and screening of human DNA samples for this variant.
SPECIFIC AIM 2 (R33 Phase): Generate a mouse knockin model bearing the Tibetan *PHD2* gene variant and obtain initial assessment of whether it is a gain of function or loss of function allele based on (1) hematocrit and red cell counts, and (2) respiratory parameters and the response of these parameters to acute hypoxia
SPECIFIC AIM 3 (R33 Phase): Assess whether the Tibetan Phd2 gene ameliorates or augments the erythrocytosis and pulmonary hypertension associated with chronic hypoxia, by (1) exposing these mice to chronic hypoxia (12% oxygen for three weeks), and (2) crossing the Tibetan *Phd2* knockin mouse with a *Hif2a* gain of function knockin (G536W) mouse that displays highly penetrant erythrocytosis and pulmonary hypertension under normoxic conditions.
Overlap: None

The Below Research support/award ended 08/31/17:
Title: Utrophin Genome Editing for Duchenne's Muscular Dystrophy (DMD) Therapy
Role: Principal Investigator
Effort: 0.2 CM
Agency: Orphan Disease Center (Pilot Grant)
Grants Officer: Samantha Charleston
Grants Officer Address: Assistant Director
Orphan Disease Center
University of Pennsylvania
Performance Period: 09/01/2016-8/31/2017
Level of Funding: $100,000
Goal: The overall aims are to develop a novel therapeutic strategy of genome editing for utrophin upregulation.
Specific Aims: Specific Aim #1: Design and optimize CRISPR-Cas9 based genome editing constructs for deleting miRNA binding sites in the 3' UTR of the mouse and human utrophin gene. Test the ability to edit and upregulate utrophin in mouse and human cell lines as well as in human induced pluripotent and muscle stem cells (hiPSCs & hMuSCs) in vitro. Specific Aim #2: Develop AAV-based vectors for achieving CRISPR-Cas9 based genome editing in mouse and human utrophin cell lines. Test the ability of AAVs to edit the endogenous gene and upregulate utrophin in mouse and human cell lines as well as in hiPSCs and hMuSCs in vitro. Specific Aim #3: Test the ability of AAV CRISPR-Cas9 based Utrophin genome editing strategy

to upreguate utrophin and rescue dystrophic pathophysiology in the mdx mouse model of DMD, in vivo.
Overlap: None

The Below Research support/award reported as pending now funded:
Title: Utrophin upregulation via let-7c SBO-mediated miRNA repression for DMD therapy.
Role: Principal Investigator
Effort: 0.75CM
Agency: Muscular Dystrophy Association
Grants Officer: Karen L. Smith
Grants Officer Address: MDA, 3300 East Sunrise Drive, Tucson AZ 85718-3299
Performance Period: 02/01/2017-01/31/2020
Level of Funding: $240,000
Goal: The overall aims are to develop utrophin upregulation as a strategy to rescue dystrophic muscle for Duchenne's Muscular Dystrophy (DMD).
Specific Aims: Specific Aim # 1. Identify and characterize utrophin 5' UTR-miRNA-mediated repression of utrophin-A:
Specific Aim # 2. Identify and develop novel utrophin 5' UTR miRNA SBOs that upregulate utrophin-A expression:
Specific Aim # 3. Test the phosphorodiamidate morpholino oligonucleotide (PMO) based let-7c miRNA SBO therapeutic strategy in *mdx* mice, *in vivo*:

The Below Research support/award reported as pending now funded:
Title: Discovery of Post-transcriptional utrophin upregulator small molecules for Duchenne Muscular Dystrophy therapeutics.
Role: Principal Investigator
Effort: 4 CM
Agency: NIH
Grants Officer: Nuckolls, Glen
Grants Officer Address: NIH/NINDS
Neuroscience Center, Room 2114
6001 Executive Blvd MSC Bethesda, MD 20892
Performance Period: 07/01/2017-06/30/2020
Level of Funding: $750,000
Goal: This project seeks to identify small molecules that increase utrophin expression for DMD therapeutics.

Specific Aims:
R21 Phase (1 Year)
Specific Aim 1: Develop a HTS assay for identification of post-transcriptional utrophin upregulation.
R33 Phase (2 Years)
Specific Aim 2: Conduct a primary HTS of the *C2C12-utrn5'luc3'UTR* assay to identify small molecules that increase utrophin expression *in vitro*.
Specific Aim 3: Prioritize / triage hits identified by HTS and validate their ability to increase endogenous utrophin protein *in vitro*.
Overlap: None

Lorberboum-Galski, Haya (Consortium- Hebrew University)
Current Research Support

Below award has ended:
Title: Pediatric Medicine – Mitochondrial Genetic Diseases: Identification & Developing Novel Treatments
Role: Principal Investigator (in collaboration with Prof. Ann Saada, Hadassah Hospital and Prof. Ophry Pines, Hebrew University)
Effort: **0%, 0 CM**
Agency: Private donation
Grants Officer: Mr. Natan Beilinson
Performance Period: 2016-**2017**
Level of Funding: 75,000$ for each researcher.
Goal: To identifying genes/mutations associated with mitochondrial diseases. In addition, to develop a combination of novel approaches to the treatment of mitochondrial disorders.
Specific Aims (my part): Developing and establishing an approach of protein replacement therapy for the treatment of mitochondrial patients. Developing a novel approach of organelle-transfer, in this case mitochondrial-transfer: The transfer of whole, healthy normal mitochondria into patients' cells defective in a mitochondrial protein.
Overlap: None

The Below Research support/award is new funding:
Title: Developing a Novel Treatment Approach for Mitochondrial Diseases
Role: Principal Investigator
Effort: 25%/ 3 calendar months
Agency: Private donation
Grants Officer: Mr. Natan Beilinson
Performance Period: 2018-2022
Level of Funding: 185,000$
Goal: Developing a novel approach of organelle-transfer, in this case mitochondrial-transfer: The transfer of whole, healthy normal mitochondria into patients' cells defective in a mitochondrial protein as well as in animal model of a mitochondrial disease
Overlap: None

The Below Research support/award is new funding:
Title: TAT-MTScs-MCM fusion proteins for the treatment of the mitochondrial disorder-methylmalonic academia (MMA)-Determine Activity of the fusion proteins
Role: Principal Investigator
Effort: 5%/ 6 calendar months
Agency: LifeMax through Yissum
Grants Officer: Mr. Natan Beilinson
Performance Period: 2018
Level of Funding: 15,000$
Goal: Production, purification and characterization of TAT-MTScs-MCM fusion protein. Determining the activity of two versions of TAT-MTScs-MCM; our fusion protein and TAT

MTScs-MCM (without the three a.a linker), by measuring reduction in MMA, in patients cells following treatment by the two fusion proteins.
Overlap: None

- **What other organizations were involved as partners?**

  Not applicable

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

  Not applicable

- **QUAD CHARTS:**

  Not applicable

9. APPENDICES

THERE ARE NO APPENDICES