AWARD NUMBER: W81XWH-17-1-0203

TITLE: Criticality of the phosphate carrier SLC25A3 for mitochondrial inorganic phosphate uptake to sustain striated muscle function

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REPORT DATE: Oct 2019

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Oct 2019	Annual	30 Sept 2018 – 29 Sept 2019

4. TITLE AND SUBTITLE

Criticality of the phosphate carrier SLC25A3 for mitochondrial inorganic phosphate uptake to sustain striated muscle function

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0203

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 the 2nd year of the project there were accomplishments for each Aim. An accomplishment benefitting all Aims was 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) determining that the PiC exerts minimal control on oxidative phosphorylation, at least in cells with fairly low ATP demand, and 3) developing the skills needed to measure, *ex vivo*, the force generation and recovery from force fatigue of mouse skeletal muscle. The main accomplishments for Aim 2 is the development of the challenging skills required to measure, *ex vivo*, cytosolic and mitochondrial Ca^{2+} fluxes in mouse skeletal muscle fibers using fluorescence microscopy. Finally, for Aim 3, the TAT-PiCA that was generated last year was 1) successfully isolated from inclusion bodies (which involved the development of a strategy to do that), 2) found to localize to the inner mitochondrial membrane mitochondria of HEK293, HeLa and HepG2 cells (the inner membrane is the endogenous location of the PiC), and 3) found to be non-toxic to 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.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	39	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. INTRODUCTION

Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

This project investigates the criticality of the mitochondrial phosphate carrier (PiC) for the two main processes within mitochondria that require inorganic phosphate, namely oxidative phosphorylation (oxphos) and buffering mitochondrial matrix Ca^{2+}. Experiments were organized into 3 Aims. Aim 1 directly investigates the required of PiC for oxphos and also investigates adaptive mechanisms that counter loss of PiC which are important to consider in the context of natural pathogenic mutations in the human gene SLC25A3 encoding PiC; these mutations lead to myopathies. Aim 2 investigates the requirement of PiC for buffering mitochondrial Ca^{2+} as well as the downstream impact on cellular Ca^{2+} signaling; indeed, mitochondria can take up a vast amount of Ca^{2+} and, as such, participate in cellular Ca^{2+} homeostasis. Changes to how mitochondria handle Ca^{2+} are expected to have a broader effect on cell functions affected by Ca^{2+}. Aim 3 turns to the potential use of a TAT fusion protein as a therapeutic tool to manage myopathies resulting from mutations in human SLC25A3. The goal of Aim 3 is to generate TAT fusion proteins for both PiC isoforms (A and B), then to determine if the fusion proteins can rescue phenotypes caused by PiC depletion. Thus this project is expected to 1) advance our basic knowledge about a fundamental process, namely supplying inorganic phosphate to mitochondria for critical functions performed by mitochondria, and 2) to generate a potential therapeutic tool for a subset of human myopathies.

2. KEYWORDS

Provide a brief list of keywords (limit to 20 words).

-Inorganic phosphate

-Mitochondrial inner membrane transporters

-Oxidative phosphorylation

-Mitochondrial calcium uptake

-TAT fusion protein

-Mitochondrial myopathy

-Mitochondrial disease

-Nutrient signaling in skeletal muscle

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.

This year we had goals that arose from several challenges that presented themselves at the end of Year 1 and during Year 2 of the grant:

1) Determine the utility of the HEK293 cells with PiC depletion obtained via CRISPR that we generated and reported on last year. This model was to be used to investigate **Aims 1 and 2, in experiments that are part of Major Task 2**. Our plan was to use these cells as a cellular model of PiC depletion that would allow mechanistic hypotheses to be more easily tested than in mice.

A lot of effort was put into generating HEK293 cells with knockout of SLC25A3 (encoding PiC) using CRISPR-CAS9. The methods and clones were described in last year's Progress Report; this information will not be repeated here except to display the immunoblot showing potential useful clones (**Figure 1**). There was the potential to generate almost 200 clones. Some clones did not display SLC25A3 knockout, some had incomplete knockout (probably because they did not arise form a single cell), and many clones died. In the end, there were only 4 clones where the cells could proliferate and that had at least 95% depletion of PiC protein.

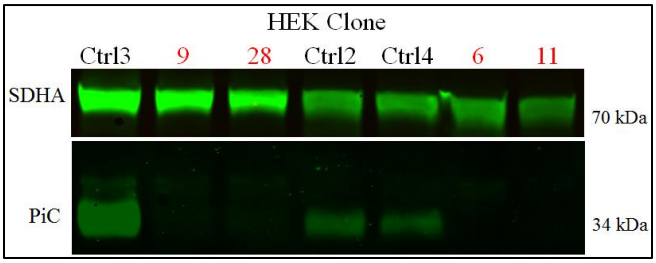

Figure 1. Immunoblot analysis of PiC expression in empty vector control HEK lines (Ctrl) and knockout lines (numbers in red). SDHA: succinate dehydrogenase, used as loading control. Whole cell lysates were used; 50ug/lane.

We performed extensive bioenergetics analyses on all 4 clones (Dr. Cesar Vasquez continued these analyses during this past year, taking over from a Ph.D. rotation student who performed them during Year 1); cells were studied intact and after permeabilized which allows analysis of maximal oxidative phosphorylation (intact cells allow analysis of only basal oxidative phosphorylation which is generally lower than the maximal rate). The end result was that oxidative phosphorylation (maximal or basal), maximal proton leak-dependent respiration and maximal electron transport chain activity were indistinguishable between clones with 95%+ depletion of PiC and Ctrl lines. Note that the measurement of oxidative phosphorylation is the key measurement because inorganic phosphate (Pi) is needed in the matrix to phosphorylate ADP; the overarching hypothesis is that PiC is required for Pi transport into the matrix to support ATP synthesis at levels needed to maintain cell viability and proliferation. The latter experiments were performed using conventional substrate conditions (i.e., 25 mM glucose, 4 mM glutamine), and also using substrate conditions that would force ATP production by mitochondria (glutamine only); results were the same. We also tested the hypothesis that the dicarboxylate transporter (DiC; exchanges Pi and succinate or malate) compensated for PiC depletion; the DiC inhibitor, butylmalonate, was used. These experiments provided no indication that the DiC was compensating for PiC depletion.

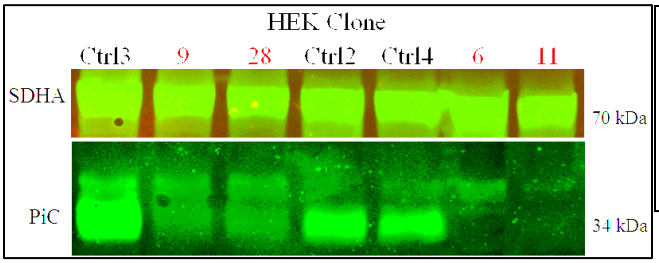

Figure 2. Immunoblot shown in Figure 1, but overexposed to show residual protein in clones 9, 28, and 11. Whole cell lysates were used; 50ug/lane.

When we overexposed the immunoblots we could clearly detect low PiC expression in the 3 of 4 clones with deep PiC knockdown (**Figure 2**). We cannot rule out that this small amount of PiC was sufficient to supply enough Pi for oxidative phosphorylation in HEK293 cells that likely derive much of their ATP from glycolysis; this would be consistent with our finding in HeLa cells that ~10% of endogenous PiC was sufficient for these cells to maintain basal levels of oxidative phosphorylation (Seifert et al., 2016). However, the hypothesis that only a very small amount of PiC is sufficient to maintain oxidative phosphorylation is difficult to test because, in our experience, siRNA cannot achieve more than 90-95% knockdown in cultured cells (Seifert et al., 2016). Furthermore, the molecule, mersalyl, that was classically used in ^{32}Pi transport studies to inhibit the high capacity rapid Pi transport in mitochondria (i.e., via PiC), is not specific when used in oxidative phosphorylation studies probably because mersalyl is an alkylating agent and thus will prevent disulphide bond formation on many molecules. I did test the effect of mersalyl on bioenergetics of control cells. The effect was to inhibit not only oxidative phosphorylation but also maximal electron transport chain activity, clearly demonstrating that mersalyl was affecting more than the PiC. So at this point we do not have adequate tools to deplete the small amount of residual PiC in CRISPRed HEK cells (or in HeLa cells with ~90% PiC depletion via siRNA).

We may generate new CRISPRed cells by a slightly different strategy. First, we would use HeLa cells, because we have previously shown that 85% depletion of PiC limits maximal oxidative phosphorylation in these cells (Seifert et al., 2016), whereas HEK cells seem to be capable of maintaining maximal oxidative phosphorylation with less PiC than HeLa cells (this project). Second, we would select single clones differently, namely by seeding cells at very low density in a 15-cm plate; the idea is that this very low seeding on a large surface would allow small colonies to grow from a single cell, and then these small colonies can be moved into individual wells of a 96-well plate. We think this strategy will optimize the possibility of obtaining populations derived from a single clone. A concern, however, is if complete PiC loss is not compatible with cell survival (see next paragraph). Generating stable knockdown (instead of knockout) HeLa cells (using shRNA) is also a strategy we

are considering; again, with HeLa cells we can take advantage of our observation that they show a bioenergetics deficit with ~10% residual PiC.

Though the outcomes of no effect of PiC depletion in HEK cells on bioenergetics parameters are less interesting than we had hoped, they support the conclusion that very little PiC is needed to maintain oxidative phosphorylation, at least in cultured cells with low ATP demand. However, we speculate that the many CRISPRed HEK clones that died were true PiC null clones, which would indicate that the PiC is required for cell survival, presumably because it is needed for oxidative phosphorylation.

That so little PiC is needed to maintain oxidative phosphorylation also indicates that the PiC would have very little control over oxidative phosphorylation; in other words, the PiC exists in much higher capacity than is needed for oxidative phosphorylation. This conclusion raises the question of the need for such a high amount of PiC. Last year's Progress report described a series of studies that tested the role of PiC to enable Ca^{2+} uptake into the mitochondrial matrix and to buffer matrix Ca^{2+}. In that report, we described experiments in the CRISPRed HEK cells that tested this role of the PiC. We found that mitochondrial Ca^{2+} uptake is in fact slightly increased and buffering of matrix Ca^{2+} is decreased, leading to elevated matrix free [Ca^{2+}].

Next steps: These experiments will be taken up again by a new post-doctoral fellow, Arijita Ghosh, who will join the lab in mid-November 2019. In addition, Dr. Cesar Vasquez has been learning how to evaluate mitochondrial and cytosolic Ca^{2+} dynamics in mouse skeletal muscle fibers so that the role of the PiC can be evaluated in our *in vivo* model of PiC depletion (See next section)

That matrix Ca^{2+} buffering is decreased by PiC depletion but not oxidative phosphorylation, in the same cell model (CRISPRed HEK cells) indicates that even if Pi is not limiting for oxidative phosphorylation, it can be limiting for Ca^{2+} buffering in the matrix, suggesting the hypothesis that the high protein levels of PiC are needed to serve the multiple roles of Pi in the matrix. Also suggested is the idea that there is a hierarchy in

the functions served by the PiC, with oxidative phosphorylation being a priority over Ca^{2+} buffering. A mechanistic possibility is that distinct pools of PiC exist in mitochondria, with much of the PiC localized nearby, or even in complex with, the adenine nucleotide translocase (that exchanges ATP and ADP), and a smaller pool that is localized near or complexed with the mitochondrial Ca^{2+} uniporter (that transports Ca^{2+} into the matrix). The latter hypotheses can also be tested, as will the implications of decreased matrix Ca^{2+} buffering on cytoplasmic Ca^{2+} homeostasis and dynamics (**Aim 2, Major Task 2**).

2) Training to perform measurements of cytosolic and mitochondrial Ca^{2+} dynamics in vivo. To determine the role of the PiC in mitochondrial Ca^{2+} uptake and buffering, we proposed and plan to undertake experiments in our mouse model with PiC depletion in skeletal muscle (**Aim 2, Major Task 4**). In last year's Progress Report we described a protocol that we developed to measure mitochondrial Ca^{2+} uptake and matrix buffering in skeletal muscle mitochondria isolated from mouse. It was also important to have a technique to perform such measurements in an intact in vivo system, in order to not only evaluate mitochondrial Ca^{2+} but also to understand the impact on cytosolic Ca^{2+} and, more generally, in cellular Ca^{2+} homeostasis. Fortunately a former post-doctoral fellow in Dr. György Hajnóczky;s lab, Dr. Veronica Eisner (not part of the grant) developed the relevant methods while in Dr. Hajnóczky's lab (Eisner et al., 2014, 2017) and visited the lab twice in the past year and thus was easily available to coach Dr. Vasquez on these very challenging methods. Specifically, Dr. Vasquez has now learned how to 1) isolate high quality skeletal muscle fibers from the *Flexor digitorum brevis* (FDB) muscle; 2) to use fluorescence microscopy to measure cytosolic Ca^{2+} using Fura2, under resting conditions; 3) to measure cytosolic Ca^{2+} changes during muscle stimulation (electrical to provoke an increase in cytosolic Ca^{2+} from sarcolemmal Ca^{2+} channels or using caffeine to provoke a release of Ca^{2+} from the sarcoplasmic reticulum); 4) to electroporate mtRCaMP into the FDB for measurements of mitochondrial matrix free [Ca^{2+}]; 5) to use fluorescence microscopy to measure the mtRCaMP signal; 6) to simultaneously measure the Fura2 and mtRCaMP signals; 7) to analyze the data.

We are happy to report that Dr. Vasquez, using practice mice from my approved IACUC

Protocol 01307, has achieved all the above except #6. He can now perform these measurements reliably, and has even further optimized some aspects of the methods to have an even higher number of successful runs on an experimental day. Furthermore, his values of resting cytosolic Ca^{2+} as well as the amplitude of the rise in cytosolic Ca^{2+} upon electrical stimulation are very similar, quantitatively, to those obtained in a study from the Hajnóczky and Seifert labs that is currently in press (DeBatistti et al. *in press in Cell Reports*; note that Dr. Eisner's studies were conducted in rats).

Next steps: These accomplishments by Dr. Vasquez are impressive, because of the difficulty of these methods, and will also be extremely useful going forward because they establish the methods in mice, and show full feasibility in Dr. Vasquez's hands. Thus Dr. Vasquez is poised to carry out the studies in mice that form part of Aim 2 (Major Task 4).

3) Training in the measurement of ex vivo skeletal muscle strength. As part of Aim 1 (Major task 3) we hypothesized that depletion of the PiC in mouse skeletal muscle would decrease force generation in skeletal muscle as well as recovery of force following from muscle fatigue, because these process rely on ATP generation by oxidative phosphorylation. These experiments harness the expertise of Sub-Awardee, Dr. Tejvir Khuarana and his post-doctoral fellow, Dr. Emanuele Loro. Dr. Loro recently left Dr. Khurana's lab for a job in industry. Over 2 months prior to Dr. Loro's departure, Dr. Loro instructed Dr. Heli Xu, a new post-doctoral fellow in my lab, as well as myself on how to perform these measurements and how to analyze the data. This was done using practice mice from my IACUC protocol 01307. Specifically, we learned how to 1) set up the force measurement instrumentation; 2) isolate high quality *Extensor digitorus longus* muscle; 3) determine the optimal muscle length that provides for maximal force generation; 4) evaluate muscle force developed in response to a twitch stimulation; 5) how to induce fatigue then to record recovery of muscle force; 6) how to weigh the muscle after the physiology experiments and then to calculate the cross sectional area of the muscle to use as a normalizing factor for the physiology measurements; 7) how to analyze the data.

Next steps: The aforementioned measurements can be successfully done by Heli and myself (though some practice is still required to obtained the highest quality muscle preparations), and therefore will be conducted in my laboratory to address Aim 1.

4) Isolating the TAT-PiC fusion protein from inclusion bodies, then determining if TAT-PiC localizes to mitochondria, and to the correct compartment of mitochondria, namely the inner mitochondrial membrane.

Last year we reported the generation of a TAT-fusion protein for PiC isoform A. When last year's Progress Report was submitted, TAT-PiC was confined to inclusion bodies, which is not surprising for a membrane protein. A major challenge, however, is to isolate TAT-PiC from inclusion bodies. That was accomplishment in Year 2, as was the successful localization of TAT-PiC to mitochondria, and specifically to the inner mitochondrial membrane (IMM). **These accomplishments, done in the lab of Sub-Awardee, Dr. Haya Loberboum-Galski, address Aim 3 (Major Task 1), and are described below**.

Modern medicine offers no cure for genetic mitochondrial disorders and the usual treatment is only palliative. We proposed in this research the concept for the treatment of mitochondrial disorders using Protein Replacement Therapy, by the delivery of a wild type mitochondrial protein directly to its sub-cellular location, in the form of a fusion protein. Our approach is to fuse the normal protein, mutated in a known mitochondrial disease, with the delivery peptide TAT, which will lead the protein into the cells and their mitochondria where it will substitute for the mutated endogenous protein or allow the expression of a protein that cannot be expressed because of a mutation. The most well-known, investigated and tested delivery peptide is the HIV-transactivator of transcription (TAT) peptide. TAT-fusion proteins are rapidly and efficiently introduced into cultured cells, intact tissue, live tissues and traverse also the mitochondrial membranes. This approach was tested by us before and proven to work, for mitochondrial matrix soluble proteins.

The approach of protein replacement was as yet not tested for mitochondrial-membrane proteins. In this research we focused on the PiC, localized to the IMM. Humans have a documented alternatively spliced exon 3A and 3B, generating two mPiC isoforms, A and

B. Thus, we designed and construct both TAT-MTS-PiCA and TAT-MTS-PiCB *human* isoforms (TAT-PiCA was described last year and is further discussed here; generation of TAT-PiC-B is underway, and not discussed here). The natural mitochondrial targeting sequence (MTS) of PiC (49 aa) was used.

Here our goal was to construct and test TAT-MTS-PiC fusion proteins as a means of rescuing deleterious phenotypes caused by mPiC loss. A schematic presentation of the fusion protein is demonstrated in **Figure 3**.

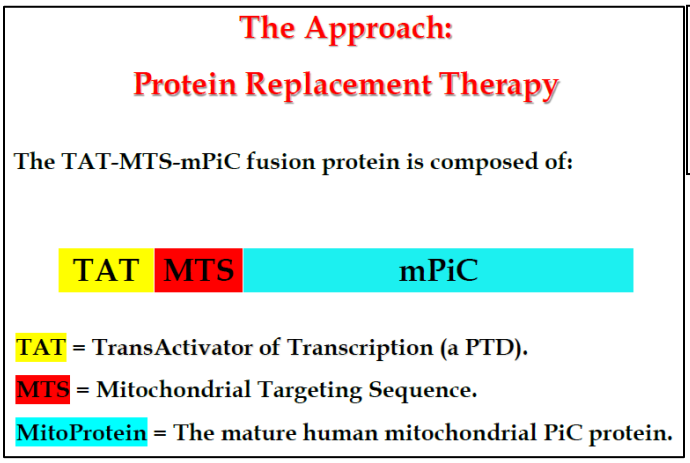

Figure 3: A schematic presentation of TAT-MTS-mPiCA fusion protein.

As reported in last year's Progress Report, calibration experiments were performed to find the bacterial host and conditions for the expression of TAT-MTS-mPiCA fusion protein (see also **Figure 6** below).

Following expression, as expected, being an inner membrane mitochondrial protein, TAT-MTSm-PiC fusion protein was mainly found in inclusion bodies (IB) of the bacterial expressing cells. Therefore, following expression, IB were separated and denatured in 6M urea. The denatured TAT-MTS-mPiC fusion proteins in 6 M Urea, were added with 10 mM imidazole. 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. Again, being a membrane protein, calibration experiments were performed to allow binding of the fusion protein to the affinity column (results not shown). A key element for

the binding to the affinity column was using NaH_2PO4 in the binding buffer. Figures 4 demonstrates a typical affinity purification of the TAT-MTS-mPiCA fusion protein.

The next crucial step was to exchange the buffer to a physiological one, allowing future biological testing of the fusion protein. Calibration experiments helped to define the

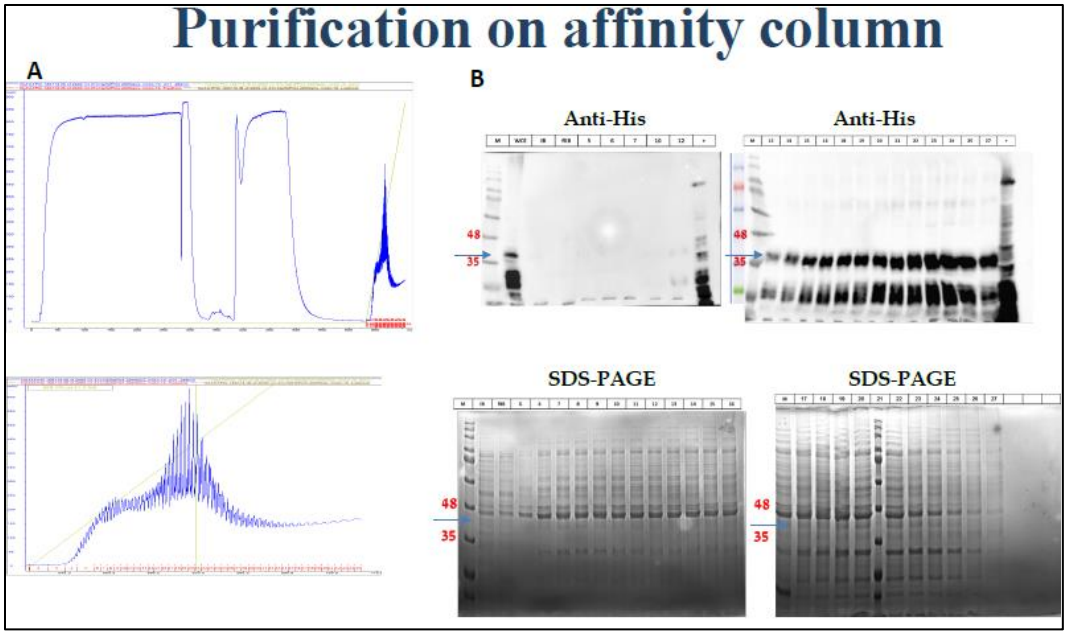

Figure 4: A Typical affinity purification of TAT-MTS-mPiCA under denatured conditions (6M Urea). **A**: Column profile; **B**: Lower panel: SDS-PAGE analysis of the affinity column fractions. Upper panel western blot analysis of the affinity column fractions using anit-His antibodies.

dialysis buffer for the partial purified protein. We found that this buffer should contain 200 mM L-Arginine (**Figure 5**). **Figure 6** summarizes all calibration experiments for purification of the TAT-MTS-mPiCA fusion protein.

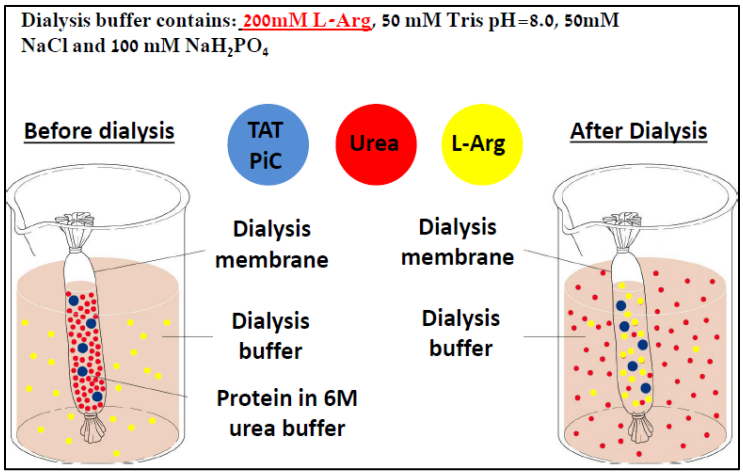

Figure 5: Schematic presentation of the dialysis step in the preparation of TAT-MTS-mPiCA fusion protein, indicating the importance of adding 200mM L-arginine to the protein preparation.

From DNA to protein

Cloning, Expression and partial Purification

Cloning

pET 28 vector

Bacterial expression system, temperature, IPTG concentration

HMS 37°c 1 mM

Lysis buffer

10mM Tris pH=8.0, 100mM NaCl, 100mM NaH_2PO_4, 10% glycerol, 2mM βME

Lysis with-

Microfluidizer

Denaturation buffer

6M Urea, 10mM Tris pH=8.0, 100mM NaCl, 100mM NaH_2PO_4, 2 mM βME

Ni Column binding buffer

"Denaturation buffer" + 5mM imidazole

Ni Column elution buffer

"Denaturation buffer" + 500mM imidazole

Dialysis

200mM L-Arg, 50 mM Tris pH=8.0, 50mM NaCl and 100 mM NaH_2PO_4

Figure 6: Summarizing conditions for purification of the TAT-MTS-mPiCA fusion protein.

Partial Purified TAT-MTS-PiCA fusion protein is not cytotoxic to cultured cells

To ensure that the TAT-MTS-mPiCA fusion protein preparation is safe to be used for further testing, we examined its effect of the viability of cultured cells, as compared to control cells exposed only to the buffer of the fusion protein. As demonstrated in **Figure 7**, TAT-MTS-mPiCA has no cytotoxic effect on the viability of HeLa cells (human cells) at various concentrations of the fusion protein.

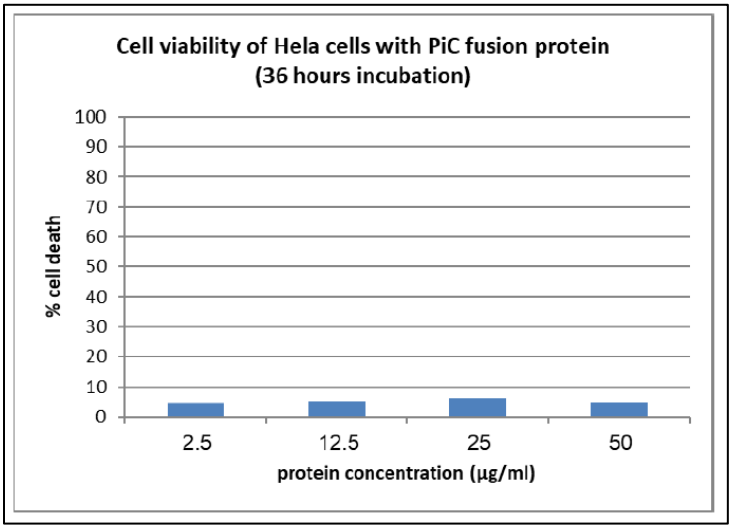

Figure 7: TAT-MTS-mPiCA fusion protein has no cytotoxic effect on the viability of human HeLa cells at various concentrations of the fusion protein.

TAT-MTS-mPiCA fusion protein is internalized into cells

We followed the internalization of the TAT-MTS-PiCA fusion protein into HeLa cells. HeLa cells were incubated with TAT-MTS-PiCA for 3 hr. We prepared then lysates from control (untreated cells) or treated cells, and followed the existence of the fusion protein using anti-PiC antibodies. As seen in **Figure 8**, the fusion protein was found in the cell lysates, and in larger amounts when added at higher concentrations (dose response). Thus, TAT-MTS-PiCA is internalized into the cells, following its incubation with cells.

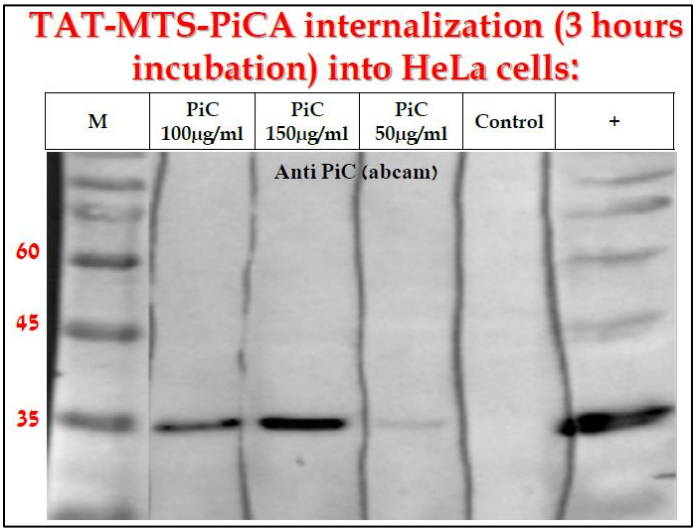

Figure 6: TAT-MTS-mPiCA fusion protein is being internalized into cells. HeLa cells were incubated with various concentration of TAT-MTS-mPiCA for 3 hr. We prepared then lysates from control (untreated cells) or treated cells, and followed the existence of the fusion protein using anti-PiC antibodies.
It should be pointed out that we used antibodies from Abcam showing the fusion protein at a MW of ~35kD (as indicated by the company itself). Using another commercial anti-PiC (Protein Tech) in next experiments identified the fusion protein at its correct ~43kD.

TAT-MTS-mPiCA added to cells reaches its endogenous location, the IMM

The major question we had to address following TAT-MTS-

mPiCA internalization into cells is, will it reach its final cellular destination, the IMM? To address this question, HeLa cells were incubated with TAT-MTS-mPiCA for 3 hr and sub-fractionation was performed (see scheme for sub-fractionation in **Figure 9**).

The existence of the fusion protein was tested by western blot analysis using anti-PiC antibodies. Purity of the sub-fractions was confirmed by using anti-Tubulin antibodies for the cytosolic sub-fraction, anti-Lamin B antibodies for the nucleus and anti-CoIV for the inner mitochondrial (IM) sub-fraction. As can be seen in **Figure 10**, TAT-MTS-mPiCA is found in the IMM with no evidence for its existence in the mitochondrial matrix or mitochondrial outer membranes + inter- membrane space.

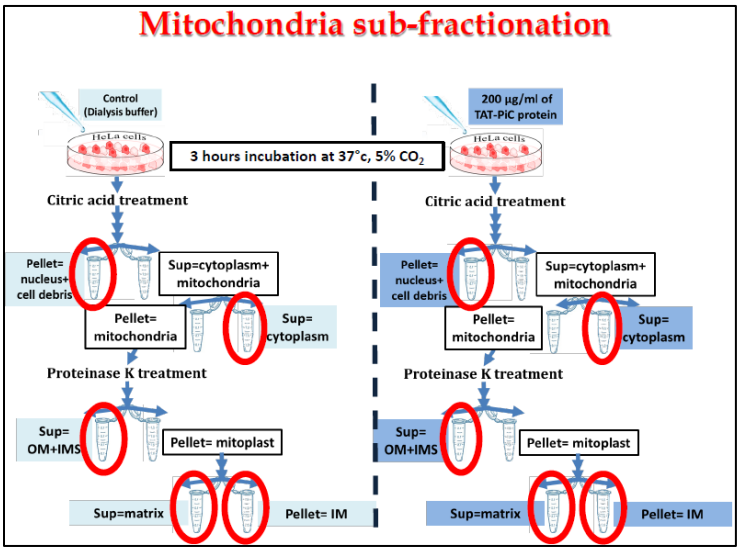

Figure 9: Schematic presentation of mitochondrial sub-fractionation.

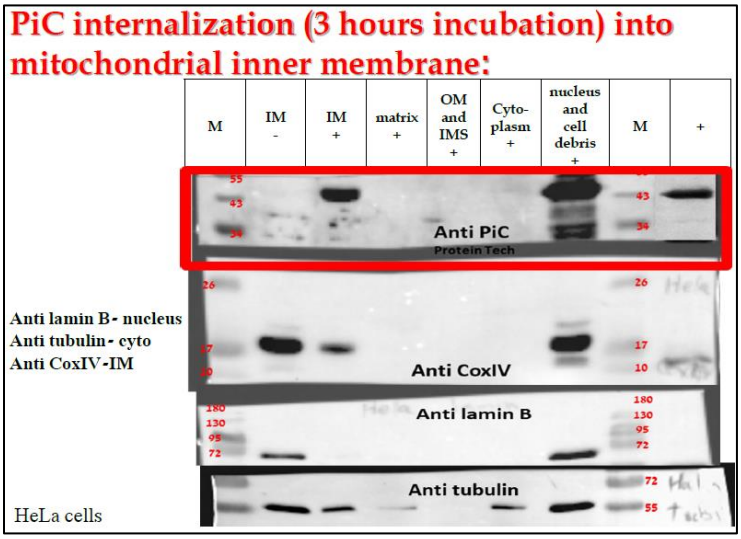

Figure 10: Localization of TAT-MTS-mPiC fusion protein within HeLa cells.
TAT-MTS-mPiCA is found in the mitochondrial inner membrane with no evidence for its existence in the mitochondrial matrix or mitochondrial outer membranes + inner membrane space.

We repeated the these experiments using 3 different cell cultures and as seen in **Figure 11**, in Hela cells, HEK293 cells and HePG2 cells incubated with the fusion protein. In all cell types, the fusion protein entered the cells and reached the IMM.

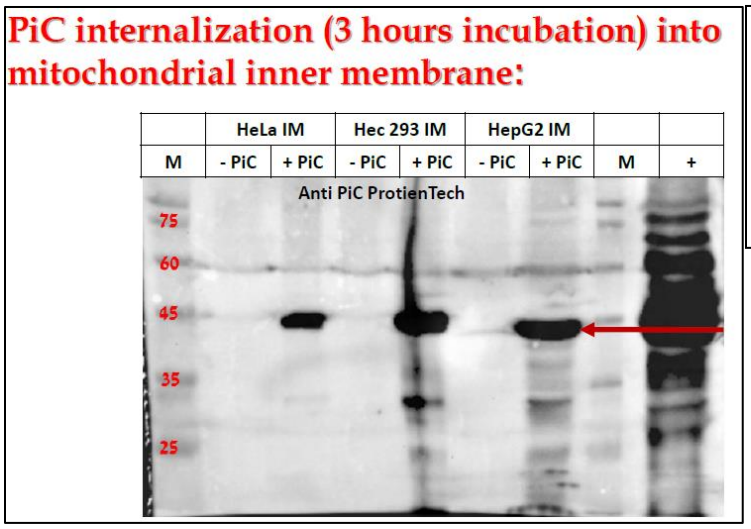

Figure 11: Localization of TAT-MTS-mPiC fusion protein within HEK293, HepG2 and HeLa cells (arrow).

Future planned experiments:

- To evaluate the functionality of TAT-MTS-mPiC in mouse and human cell models depleted in PiC, using ^{32}Pi transport assays and bioenergetics studies
- To generate a knockout cell line using CRISPR/Cas9 technology and to test the ability of TAT-MTS-mPiCA to be delivered into SLC25A3 knockout cells and into its mitochondrial inner membrane.
- Measuring cell viability, proliferation rate, bioenergetics (by Seahorse, ATP assay), inspection of mitochondrial network (MitoTracker), and mitochondrial Ca^{2+} uptake in cells treated with TAT-MTS-mPiCA.
- To test the *in vivo* effects of TAT-PiC treatment in a PiC deficient mouse model.

Part of these planned experiments, mainly the *in vivo* studies, will be performed at the Seifert lab.

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phosphate carrier loss: differential dependence of mitochondrial metabolism and dynamics, and cell survival, on the extent of depletion. *J Biol Chem*. 291:26126-26137. PMID 27780865. *Co-corresponding authors.

Eisner V, Lenaers G, Hajnóczky G. (2014) Mitochondrial fusion is frequent in skeletal muscle and supports excitation-contraction coupling. *J Cell Biol*. 205: 179-95. PMID: 24751540

Eisner V, Cupo RR, Gao E, Csordás G, Slovinsky WS, Paillard M, Cheng L, Ibetti J, Chen SR, Chuprun JK, Hoek JB, Koch WJ, Hajnóczky G. (2017) Mitochondrial fusion dynamics is robust in the heart and depends on calcium oscillations and contractile activity. *Proc Natl Acad Sci U S A*. 114: E859-E868. PMID: 28096338

4. IMPACT

- *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
- **What was the impact on the development of the principal discipline(s) of the project?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).*

-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?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an 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**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:*
        - *transfer of results to entities in government or industry;*
        - *instances where the research has led to the initiation of a start-up company; or*
        - *adoption of new practices.*
- **What was the impact on society beyond science and technology?**
    - *If there is nothing significant to report during this reporting period, state "Nothing to Report."*
    - *Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:*
        - *improving public knowledge, attitudes, skills, and abilities;*
        - *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
        - *improving social, economic, civic, or environmental conditions.*

-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. This project has already benefitted several trainees, namely Dr. Cesar Vasquez (post-doctoral fellow, Seifert lab) who has now had extensive training in the measurements of Ca^{2+} fluxes and in microscopy, Ms. Samar Zabit (PhD student, Dr. Loberboum-Galski's lab) in recombinant protein production and purification, and cellular subfractionation, Dr. Valentina DeBatistti in Ca^{2+} flux studies in cells (Hajnóczky and Seifert labs), and Dr. Heli Xu, a relatively new post-doctoral fellow in the Seifert lab who has now had training to evaluate skeletal muscle physiology, which nicely complements her prior training, from her PhD work, on skeletal muscle metabolism.

5. CHANGES/PROBLEMS

The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

- **Changes in approach and reasons for change**
  - *Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.*

No changes in the approach

- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - *Describe problems or delays encountered during the reporting period and actions or plans to resolve them.*

The mouse work was delayed. The ACURO has now been approved.

- **Changes that had a significant impact on expenditures NO CHANGES**
  - *Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.*
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Please see the above paragraph addressing significant delays.

  - *Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.*
- **Significant changes in use or care of human subjects. NO CHANGES**
- **Significant changes in use or care of vertebrate animals. NO CHANGES**
- **Significant changes in use of biohazards and/or select agent NO CHANGES**

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

- *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*
- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.
    - **Journal publications.** *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).* **No. NOTHING TO REPORT**
    - **Books or other non-periodical, one-time publications.** *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).* **No. NOTHING TO REPORT**
    - **Other publications, conference papers, and presentations.** *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.* **No. NOTHING TO REPORT**
- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section. **NOTHING TO REPORT**
- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.
  NOTHING TO REPORT
- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award. **NOTHING TO REPORT**
- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

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:	3
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 Drs. Cesar Vasquez and Heli Xu, as well as Briyanna Hymms (technician), and also Aishwaryan Sivaranakrishman (until her departure for Grad School this past July) 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)
Funding Support:	NA

Name:	Cesar Vasquez
Project Role:	Post Doc fellow

Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	9
Contribution to Project:	Cesar Vasquez has performed work in the area of Aim 2 (Ca2+ flux studies)
Funding Support:	NA

Name:	Heli Xu
Project Role:	Post Doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	Heli Xu has performed work in the area of Aim 1, specifically the skeletal muscle physiology studies
Funding Support:	NA

Name:	Briyanna Hymms
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	Briyanna Hymms has performed work in the area of Aim 1 and 2. Her role is to assist Drs. Vasquez and Xu.
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) Terminated
Funding Support:	NA

Name:	Noa Hauzer
Project Role:	Student (Hebrew University/Consortium)
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	12
Contribution to Project:	Noa Hauzer has performed work by carrying out the planned experiments; including cloning of the TAT-MTS-mPiC (both two mPiC isoforms-A&B); expression in bacterial hosts, purification of the fusion proteins, testing the proteins on cultured cells regarding its internalization into the cells and into its final destination; the mitochondrial inner membrane, biological activity as phosphate carrier and its effect on cell and mitochondrial function.
Funding Support:	NA

Name:	Samar Zabat
Project Role:	Student (Hebrew University/Consortium)
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	12
Contribution to Project:	Samar Zabat has performed work by carrying out the planned experiments; including cloning of the TAT-MTS-mPiC (both two mPiC isoforms-A&B); expression in bacterial hosts, purification of the fusion proteins, testing the proteins on cultured cells regarding its internalization into the cells and into its final destination; the mitochondrial inner membrane, biological

	activity as phosphate carrier and its effect on cell and mitochondrial function.
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, training 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:	3
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, training 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 Active Research Support:

The Below Research support/award level of funding has been updated:
Title: Molecular Mechanisms of Mitochondrial Ca2+ Transport
Identifier: R01 GM102724
Role: Co-Investigator
Effort: .36 CM
Agency: NIH/NIGMS
Grants Officer/Address: Eileen Hyde, Hydee@nigms.nih.gov
Performance Period: 02/01/15-02/28/2021
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.

The Below Research support/award end date has been updated:
Title: Impact of Frataxin deficiency on cardiac substrate metabolism
Role: Principle Investigator
Effort: .24 CM
Agency: Friedreich Ataxia Research Alliance (FARA)
Grants Officer/Address: Jennifer Farmer
Performance Period: 09/01/2016-**12/31/19**
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 level of funding has been updated:
Title: Regulation of substrate metabolism in skeletal muscle by mitochondrial thioesterases
Identifier: R01 DK109100
Role: Principal Investigator
Time Commitment: 2.4 CM
Supporting Agency: NIH
Contracting/Grants Officer: Craig Bagdon, bagdonc@niddk.nih.gov
Performance Period: 7/01/17-6/30/21
Level of Funding: **$264,723**
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.

Title: Pathogenesis of Myopathies Caused by Novel Mitochondrial Phosphate Carrier Mutations
Identifier: R01GM123771
Role: Principle Investigator
Effort**:** 3 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.

Title: Study of the mitochondrial-cellular response to environmental stress by flourescence imaging
Identifier: R21 ES025672/4R33 ES025672

MPI: Hajnoczky & Csordas
Role: Co-Investigator
Time Commitment: .24 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

Title: Molecular composition of the mitochondrial calcium uniporter and cardiac pathophysiology
Identifier: R01 HL142271
Hajnoczky & Elrod –MPI
Role: Co-Investigator
Time Commitment: .24 CM
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

Hajnoczky, Gyorgy
Current Research Support

The Below Research support/award has ended
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 has ended
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 calendar months and award level of funding has been updated:
Title: Molecular Mechanisms of Mitochondrial Ca2+ Transport
Identifier: R01 GM102724
Hajnoczky PI
Time Commitment: **1.56** 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: **$208,983**
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 level of funding has been updated.
Title: Redox regulation of intracellular calcium signaling
Identifier: R01 DK103558
MPI: Hajnoczky & Joseph
Time Commitment: .12 Calendar Months
Supporting Agency: NIH/NIDDK
Contracting/Grants Officer: Craig Bagdon, bagdonc@niddk.nih.gov
Performance Period: 4/01/2015 – 3/31/**20 NCE**
Level of Funding: **$2,332.00 Salary Support only**
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 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: 1.92 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: **$168,042 Hajnoczky direct portion**
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 level of funding has been updated.
Title: Pathogenesis of Myopathies Caused by Novel Mitochondrial Phosphate Carrier Mutations
Identifier: R01 GM123771
Role: Co- Investigator
Effort: .48 Calendar months
Agency: NIH
Grants Officer/Address: Vernon Anderson, Ph.D.
Performance Period: 09/01/17-08/31/21
Level of Funding: **$11,903 Salary Support Only**
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 level of funding has been updated.
Title: Molecular composition of the mitochondrial calcium uniporter and cardiac pathophysiology
Identifier: R01 HL142271
Hajnoczky & Elrod -MPI
Time Commitment: 2.16 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: **$351,437 Hajnoczky direct portion**
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

Title: (PQ5) Relevance of VDAC2 heterogeneity for hepatic tumor growth and targeting
Identifier: R01 CA216254
Hajnoczky PI
Time Commitment: 2.16 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

The Below Research support/award is new funding
Title: Targeting Mitochondria To Treat Heart Disease
Hajnoczky PI
Time Commitment: .12 Calendar Months
Supporting Agency: Fondation Leducq
Contracting/Grants Officer: David Tancredi, Executive Director
Performance Period: 04/01/19-12/31/21
Level of Funding: $96,819
Project Goals/Specific Aims: This project aims to develop and test means to mitigate the ischemia-reperfusion-induced cardiac injury by interfering with mitochondrial calcium overload. We will study the role of the MICU-MCU interface in the effect of various drugs that target mitochondrial calcium transport. We will also employ new fluorescent protein based calcium and reactive oxygen species sensors for measuring the signaling activity of single mitochondria.
Overlap: None

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

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

The Below Research support/award has ended:
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

The Below Research support/award has ended:
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

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 level of funding has been updated:
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: **$1,207,500**
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

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

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: 2019-2020
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:** *For collaborative awards, independent reports are required from **BOTH** the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.*
- **QUAD CHARTS:** *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

NOT APPLICABLE.

9. APPENDICES

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

NOTHING TO REPORT