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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. In this reporting period (Nov 2019 Æ Oct 2020), substantial progress was made on Aims 2 and 3. For Aim 2, we have almost completed a manuscript that investigates the role of the PiC in mitochondrial Ca ²⁺ handling and the implications on cytosolic Ca ²⁺ signaling in muscle fibers and force generation of intact muscle. These studies utilized a new mouse model in which PiC is deleted specifically in skeletal muscle (skm), in a Tamoxifen-inducible fashion. For the experiments, PiC was deleted by injecting 9-week old mice with Tamoxifen; skm mitochondria, intact fibers and whole muscle were harvested 2 weeks later at which point PiC protein was only ~3% of normal levels. The main findings are summarized as follows. 1) Even very low PiC levels were sufficient to maintain normal mitochondrial Ca ²⁺ uptake. 2) A larger PiC capacity then was needed to support normal mitochondrial Ca ²⁺ uptake was needed to provide sufficient Pi to the mitochondrial matrix to					
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1. INTRODUCTION

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

- 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.

Overview:

During the past 2 years (Progress Reports 2018 and 2019) we focused heavily on **Aims 1 and 3**. Progress Report 2019 also presented experiments addressing **Aim 2**.

Aims 1 and 2. We had generated a cell model with PiC knockout (KO) via CRIPR, described in Progress Report 2018. Progress Reports 2018 and 2019 detailed our efforts to search for phenotypes in the HEK PiC KO cells; specifically, we extensively searched for oxidative phosphorylation (oxphos) and calcium transport/signaling phenotypes. It was clear that the model did not possess an oxphos phenotype (Progress report 2018). However, a calcium phenotype seemed present (Progress Report 2019). **During the past year** we sought to reproduce the calcium findings, using new aliquots of cells and also KO cells generated using different guide RNAs. However we could not reliably repeat the findings we reported in Progress Report 2019. Using the new KO cells, we also attempted additional experiments, using permeabilized cells, to try to specifically understand if mitochondrial Ca^{2+} uptake and matrix free Ca^{2+} were, respectively, decreased and increased by PiC loss (as was hypothesized). However, despite numerous attempts that included several modifications to the experimental design (e.g., in permeabilized cells, depleting mitochondria of inorganic phosphate), we could not detect any differences between Control and PiC KO cells. Our conclusion is that either a small amount of PiC remains in “KO” cells (though successive passages of KO cells did not lead to a clear expression of PiC on western blot, as would be predicted if a few cells with normal PiC “contaminated” early passage cells), and/or that alternative inorganic phosphate (Pi) transporters exist to uptake Pi into mitochondria, and/or that there is adequate substrate-level phosphorylation/provisioning of Pi within the mitochondrial matrix. Indeed, our study (1) demonstrated that HeLa cells required >85% depletion of PiC for a decrease in maximal oxphos to become evident. That >>85% depletion of PiC in CRISPRed HEK did not lead to a suppression of maximal oxphos in HEK cells clearly indicates that different compensatory

mechanisms are available in HeLa and HEK cells. **All things considered, because we cannot detect reliable oxphos or Ca^{2+} phenotypes in HEK PiC KO cells, and have had much more success with the mouse model (see below), we refocused our efforts on the mouse model.**

Aim 3. As presented in Progress Reports 2018 and 2019. In particular, Dr. Lorberboum-Galski and her student were able to generate and purify a TAT-fusion PiC protein and demonstrate its correct mitochondrial localization. **This was a major accomplishment; it represents, to our knowledge, the first successful generation of TAT-fusion protein for a membrane-spanning protein.** The next step, and what was to be the focus of the past year, was to generate quantities of the protein to allow for further testing, namely 1) to test that TAT-PiC was functional in cells, and 2) that TAT-PiC could rescue phenotypes associated with PiC deficient. These plans were derailed by the worldwide pandemic that resulted in substantial and forced time away from the lab, in both the US (Seifert lab) and Israel (Lorberboum-Galski lab). Moreover, we did not want to risk sending precious samples of the TAT-PiC via courier and having the package get lost. Therefore, some planned activities for Aim 3 have been delayed and will hopefully be feasible soon (during the No Cost Extension phase of the grant). However, **as shown below, progress has been made to test TAT-PiC in cells with PiC knockdown.**

What follows addresses Aims 2 and 3.

Aim 2

The following represents substantial progress towards completion of a project. We anticipate that a manuscript will be submitted by the end of December 2020.

Aim 2: To test if PiC deficiency causes dysregulation of cytoplasmic and mitochondrial Ca^{2+} and Ca^{2+} regulated functions.

The model we used was a mouse with PiC depletion from skeletal muscle (via the Human Skeletal Actin promoter, only expressed in skeletal muscle (2); **Fig 1A: ALL figures are at the end of the report, after references**), induced by Tamoxifen (30 mg/kg intraperitoneally for 5 days). Mice were studied 14 days after the last Tamoxifen injection. PiC was substantially depleted from skeletal muscle mitochondria (**Fig 1B**) but not from other tissues such as the heart (not shown). The extent of PiC depletion (PiC Kd) from skeletal muscle mitochondria was ~97% (**Fig 1B**).

To evaluate the impact of PiC depletion on Ca^{2+} uptake into mitochondria and the matrix buffering of Ca^{2+} , mitochondria were isolated from skeletal muscle of Ctrl and PiC Kd mice two weeks after the last Tamoxifen injection. PiC activity was predicted to affect both Ca^{2+} delivery to the mitochondria and Ca^{2+} chelation inside the matrix (3-8). Thus we recorded changes in buffer $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_c$: clearance of buffer Ca^{2+} reflects *total* Ca^{2+} uptake by mitochondria; thapsigargin was used to inhibit uptake of Ca^{2+} by SERCA into any remaining sarcoplasmic reticulum) elicited by adding a bolus of CaCl_2 and, in parallel, measured the corresponding $[\text{Ca}^{2+}]_m$ (mitochondrial matrix *free* Ca^{2+}). Assessment of matrix *free* calcium concentration changes as a function of Ca^{2+} clearance from the cytoplasmic buffer (i.e., *total* uptake) allowed the comparison of matrix Ca^{2+} *buffering* as a function of total Ca^{2+} uptake (determined by Ca^{2+} clearance, i.e., change in $[\text{Ca}^{2+}]_c$). We previously used this approach to show that cells depleted of the MICU1 component of the mitochondrial Ca^{2+} uniporter show a greatly suppressed matrix *free* Ca^{2+} rise relative to control for the same cytoplasmic Ca^{2+} clearance as control; this indicated enhanced matrix Ca^{2+} buffering capacity in MICU depleted cells (9).

In all experiments, isolated mitochondria were depleted of residual inorganic phosphate (Pi) by incubating mitochondria with glucose, hexokinase, ATP and pyruvate malate, as in (4-6). To measure $[\text{Ca}^{2+}]_m$ (matrix *free* $[\text{Ca}^{2+}]$), Pi-depleted mitochondria were loaded with FURA2-AM Ca^{2+} indicator dye ($K_d \sim 224$ nM for Ca^{2+} ; ratiometric), spun then resuspended in recording medium devoid of FURA-AM. Ca^{2+} clearance from the buffer $[\text{Ca}^{2+}]_c$ was measured using either RhodFF ($K_d \sim 2$ μM for Ca^{2+} ; single wavelength) when measured simultaneously with $[\text{Ca}^{2+}]_m$, or using FURA-FF-FA ($K_d \sim 10$ μM for Ca^{2+} ; ratiometric) when measured simultaneously with membrane potential ($\Delta\psi$: $\Delta\Psi_m$) using TMRM. Mitochondria were energized using either pyruvate/malate (5/5 mM) or succinate/rotenone (10 mM/1 μM : rotenone was used to prevent backflow of electrons through Complex I that would lead to the generation of superoxide).

The first question we addressed was whether skeletal muscle (skm) mitochondria depleted of PiC were capable of taking up Ca^{2+} ; the prediction was that mitochondrial Ca^{2+} uptake would be curtailed in the absence of PiC, because PiC is predicted to be the main or only means of Pi transport into the mitochondrial matrix, and the total amount of Ca^{2+} that can be taken up into mitochondria depends strongly on Pi (7), though not the initial rate of Ca^{2+} uptake which is independent of Pi (3). To this end, skm mitochondria were suspended in recording medium in a stirred cuvette, at 37°C , to which 10 μM CaCl_2 . As shown in **Fig 2A** and **Fig 2B**, CaCl_2 addition lead to a rapid increase in the RhodFF signal followed by a steep decrease in that signal indicating Ca^{2+} uptake by the mitochondria. The RhodFF signal was inhibited by Ruthenium Red (RuRed, inhibitor of mitochondrial Ca^{2+} uniporter), indicating that the RhodFF signal reflected Ca^{2+} uptake by mitochondria. The RuRed-inhibitable RhodFF signal was robust in both Ctrl and PiC Kd, and occurred whether mitochondria were energized with pyruvate and malate (**Fig 2A**) or succinate/rotenone (**Fig 2B**). In fact, Ca^{2+} clearance with pyruvate/malate energization was slightly greater in PiC Kd mitochondria (**Fig 2A**). Similar results were obtained whether $[\text{Ca}^{2+}]_c$

was measured using RhodFF or FURA-FF-FA. Thus we conclude that mitochondrial Ca^{2+} uptake can proceed in almost the complete absence of the PiC.

Matrix Pi, by binding matrix Ca^{2+} to form precipitates composed of calcium and phosphorus (mainly: $(\text{Ca})_3(\text{PO}_4)_2$, octacalcium phosphate, CaHPO_4 , and apatite (6)). These calcium-phosphate precipitates buffer matrix Ca^{2+} , thereby decreasing the pool of free Ca^{2+} in the matrix, and also allow mitochondria to accumulate a vast amount of Ca^{2+} (5, 6, 8). Limited Pi uptake into mitochondria would therefore predict that, for the same mitochondrial Ca^{2+} uptake, free Ca^{2+} inside the matrix will be higher than when ample Pi is taken up into mitochondria. This was investigated by adding 10 μM CaCl_2 to the cuvette containing mitochondria, then measuring $[\text{Ca}^{2+}]_m$ using FURA-AM (**Fig 2C, D**) simultaneous with the $[\text{Ca}^{2+}]_c$ measurements by RhodFF (**Fig 2A, B**). In mitochondria energized using pyruvate/malate, $[\text{Ca}^{2+}]_m$ was substantially higher in PiC KD mitochondria (**Fig 2C**). This was also the case when mitochondria were energized using succinate/rotenone, although the difference was less (**Fig 2D**).

Though experiments shown in **Fig 2C** and **Fig 2D** suggest that matrix free Ca^{2+} is indeed higher in PiC Kd mitochondria, another possibility is that a greater Ca^{2+} uptake in PiC Kd mitochondria energized with pyruvate/malate is responsible for the higher matrix free Ca^{2+} levels. To test this possibility, the mitochondrial Ca^{2+} uniporter (mtCU), the main or only uptake route for Ca^{2+} into mitochondria, was partially inhibited by Ruthenium Red (5 nM RuRed) in PiC Kd mitochondria such that Ca^{2+} clearance was similar or even less than in Ctrl mitochondria (**Fig 2E**). In this situation, upon 10 μM CaCl_2 addition, $[\text{Ca}^{2+}]_m$ remained higher in PiC Kd mitochondria (**Fig 2F**). Thus, for the same (or lesser) amount of Ca^{2+} taken up into mitochondria, PiC Kd mitochondria had a higher free $[\text{Ca}^{2+}]$ in the matrix. This observation indicates the importance of the PiC for lowering matrix free Ca^{2+} .

Ca^{2+} uptake into mitochondria requires the driving force of the membrane potential ($\Delta\Psi_m$). $\Delta\Psi_m$ measurement was also of interest in light of the greater Ca^{2+} uptake into PiC Kd mitochondria energized with pyruvate/malate (**Fig 2A**), as this greater uptake could be due to a greater driving force. Thus $\Delta\Psi_m$ was measured using TMRM, simultaneous with $[\text{Ca}^{2+}]_c$ measurements (using FURA-FF-FA) (**Fig 3A, B**). Addition of 10 μM CaCl_2 to the cuvette containing mitochondria lead to membrane depolarization in both Ctrl and PiC Kd mitochondria, regardless of the substrate used to energize the mitochondria (**Fig 3A, B**). The characteristics of the depolarization were substrate- and genotype-dependent. Depolarization was sustained when pyruvate/malate was the substrate and transient when succinate/rotenone was used (compare **Fig 3B** and **Fig 3A**, quantification: “10 Ca^{2+} 210 s” and “10 Ca^{2+} 255 s”). With both substrates, an early depolarization was greater in PiC Kd mitochondria, and this greater depolarization was sustained with pyruvate/malate (**Fig 3A**: quantification: see both “210 s – Before Ca^{2+} ” and “355 s – Before Ca^{2+} ”). Altogether these observations indicate that the greater Ca^{2+} uptake by PiC Kd mitochondria was not due to a greater driving force for Ca^{2+} uptake during the uptake phase.

To better understand the greater Ca^{2+} uptake by PiC Kd mitochondria energized with pyruvate/malate (**Fig 2A**), we questioned if there was a greater oxidative phosphorylation-related depolarization of Ctrl mitochondria prior to CaCl_2 addition that would decrease the driving force for subsequent mitochondrial Ca^{2+} uptake. To this end, we first note that, upon addition of mitochondria to the cuvette that already contained substrates (but not yet CaCl_2), $\Delta\Psi_m$ was similar between Ctrl and PiC Kd mitochondria (**Fig 3A, B**: see “Before Ca^{2+} ”). Because some of this $\Delta\Psi_m$ might reflect oxidative phosphorylation (oxphos; due to residual adenine nucleotides and some Pi in the mitochondrial matrix, despite the use of the Pi depletion protocol), we also evaluated $\Delta\Psi_m$ after addition of oligomycin to inhibit the ATP synthase and to thereby inhibit ADP phosphorylation. Oligomycin would also prevent any ATP hydrolysis from occurring. In both Ctrl and PiC Kd mitochondria (energized with pyruvate/malate), addition of oligomycin prior to CaCl_2 lead to a small but clear hyperpolarization (**Fig 3C**: compare “before oligo” and “after oligo before Ca^{2+} ”), indicating that some oxphos was occurring in mitochondria from both genotypes; the extent of the hyperpolarization was similar in Ctrl and PiC Kd (**Fig 3C**: see “after oligo before Ca^{2+} ”). Subsequent addition of 10 μM CaCl_2 lead to a rapid clearance of Ca^{2+} by mitochondria (**Fig 3D**), as occurred without oligomycin (**Fig 2A**). Addition of oligomycin did not substantially alter the Ca^{2+} clearance rate in either genotype (**Fig 3D**), indicating that Ca^{2+} clearance differences between Ctrl and PiC Kd mitochondria are unlikely to be explained by differences in oxphos-induced hyperpolarization prior to CaCl_2 addition, or to ATP hydrolysis.

Because the availability of mtCU will affect the mitochondrial Ca^{2+} uptake rate, we evaluated the expression level of the components of the mtCU, namely MCU (the pore-forming subunit), and MICU1 and MICU3, the main mtCU regulators expressed in skm. For the regulator MICU2, only 1 or no peptides was detected, which was expected because MICU2 expression is known to be low in skm. Presented here are proteomics data obtained by performing long-read-time mass spectroscopy on isolated skm mitochondria. The values were normalized to the average of 34 highly expressed proteins that did not differ between Ctrl and PiC Kd by more than 1%. Data show that MCU is upregulated in PiC Kd mitochondria (**Fig 3E**); this did not reach significance and measurements will be repeated using immunoblotting with a validated antibody against MCU. We could not detect EMRE, the scaffold protein required for mtCU function, by mass spectroscopy, likely because EMRE is very small (15 kDa); EMRE protein expression to be evaluated using a validated antibody. Unless EMRE is decreased, the greater expression of MCU in PiC Kd mitochondria can explain the greater Ca^{2+} uptake rate in PiC Kd mitochondria.

Because the greater sustained depolarization with Ca^{2+} uptake into PiC Kd mitochondria energized with pyruvate/malate was so pronounced (**Fig 3A**), we sought to further explore it. Permeability transition pore (PTP) opening (generation of a large pore in the inner mitochondrial membrane), leading to loss of $\Delta\Psi_m$, matrix swelling and eventually rupture of the outer mitochondrial membrane, loss of cytochrome c and apoptosis, is favored by elevated matrix Ca^{2+}

and Pi (10). To determine if the greater depolarization observed upon 10 μM CaCl_2 addition to PiC Kd mitochondria reflected PTP opening, we repeated the experiment in the presence of the PTP inhibitor cyclosporine A (CsA). CsA did not prevent the greater depolarization of PiC Kd mitochondria upon Ca^{2+} uptake (**Fig 4A**). To further explore a possible role for PTP in Ca^{2+} -induced depolarization in PiC Kd mitochondria versus Ctrl mitochondria, PTP was stimulated by adding pulses of 10 μM CaCl_2 to mitochondria suspended in medium free of adenine nucleotides. $[\text{Ca}^{2+}]_c$ and $\Delta\Psi_m$ were evaluated. CaCl_2 pulses (10 μM each) were initially taken up by mitochondria, but eventually uptake is no longer possible (**Fig 4B**). Moreover, $\Delta\Psi_m$ underwent a large depolarization such that $\Delta\Psi_m$ almost reached the level observed after addition of the chemical uncoupler, FCCP (**Fig 4B**). This abrupt loss of $\Delta\Psi_m$ reflects opening of the PTP, and is inhibited by CsA (**Fig 4B**). PiC Kd mitochondria also undergo CsA-inhibitable PTP; however, more CaCl_2 are required (**Fig 4B**), indicating that PiC Kd mitochondria are less sensitive to undergoing PTP. This was also observed in heart mitochondria depleted of PiC (11), and supports previous studies suggesting that the PiC (or Pi) promotes PTP opening (10, 12). We will perform more replicates of this experiment; if the result shown in **Fig 4B** holds, then this will indicate that PTP opening is unlikely to explain any greater Ca^{2+} -depolarization in PiC Kd mitochondria.

The experiments with oligomycin (**Fig 3C**) provided insight into the greater depolarization upon 10 μM CaCl_2 addition to PiC Kd mitochondria energized with pyruvate/malate. Measurement of $\Delta\Psi_m$ in those experiments revealed that oligomycin diminished the CaCl_2 -induced depolarization mainly in PiC Kd mitochondria (**Fig 3C**: compare “After oligo before Ca^{2+} ” and “After oligo after Ca^{2+} ”), and this did not reflect lesser mitochondrial Ca^{2+} uptake by either genotype in the presence of oligomycin (**Fig 3D**).

The almost absent Ca^{2+} -induced depolarization in the presence of oligomycin (**Fig 3C**) may reflect the inhibition of Ca^{2+} -induced oxidative phosphorylation. Indeed, an expected role for mitochondrial matrix free Ca^{2+} is to stimulate oxidative phosphorylation, via a direct effect of Ca^{2+} on dehydrogenases of the Krebs cycle (isocitrate dehydrogenase and α -ketoglutarate dehydrogenase: Ca^{2+} lowers the K_m for substrate (13)), and on the pyruvate dehydrogenase phosphatase (PTP1; Ca^{2+} increases the V_{max} (13)). Ca^{2+} -induced stimulation of PTP1 activity would lead to the dephosphorylation of pyruvate dehydrogenase (PDH), leading to greater activity of PDH and thus increased conversion of pyruvate to acetyl-CoA and its entry into the TCA. To further test if Ca^{2+} -induced oxphos contributes to the greater Ca^{2+} -induced depolarization of PiC Kd, the extent of depolarization upon Ca^{2+} addition will be tested using different substrates that would allow for a Ca^{2+} stimulated component (glutamate/malate and α -ketoglutarate: Krebs cycle dehydrogenases are required for the oxidation of these substrates) and substrates that would not involve a Ca^{2+} -stimulated component (duroquinol, which feeds electrons directly into ubiquinone then into Complex III when Complex I and II are inhibited; and TMPD, which feeds electrons directly into Complex IV when Complex III is inhibited). These additional experiments will be included in Fig 4. Additional experiments will also be

conducted in which Pi is absent from the assay medium then added back, in the absence and presence of oligomycin. These additional experiments are expected to better resolve and the Ca^{2+} depolarization in PiC Kd mitochondria and to understand to what extent it reflects increased oxidative phosphorylation. This latter point is of interest to understand because it addresses the possibility that calcium-phosphate precipitate formation can regulate the amount of Ca^{2+} available to stimulate oxidative phosphorylation.

Because mitochondrial Ca^{2+} uptake can shape cytoplasmic Ca^{2+} signals, we determined if PiC depletion would have an impact on cytoplasmic Ca^{2+} signals. To investigate this, we isolated fibers from the Flexor digitorum brevis (FDB). Cytoplasmic Ca^{2+} was measured in isolated fibers using protocols developed in the lab of György Hajnóczky (Key Personnel on the grant)(14, 15). Briefly, FDB was dissected from mice, digested using collagenase to loosen the fibers, then individual fibers were attached to coverslips using Cell-Tak, then incubated in DMEM/F12 medium (containing 5 mM glucose and 4 mM glutamine as substrates). Cytoplasmic Ca^{2+} was measured using FURA2-AM; fibers were loaded with FURA2-AM by incubating them for 20 mins with the dye along with 0.003% pluronic acid and 100 μM sulfinpyrazone, at room temperature. For recordings, an extracellular medium containing 2% bovine serum albumin and glucose as the substrate was used, supplemented with N-benzyl-p-toluene sulphonamide (a type II actomyosin inhibitor) to inhibit skm contraction (which would cause fibers to detach from overslips). To provoke Ca^{2+} uptake and intracellular Ca^{2+} signaling, fibers were challenged with two stimulation protocols: a single twitch protocol (15) and a tetanic stimulus protocol (14, 15).

Baseline “resting” $[\text{Ca}^{2+}]_c$ measured prior to muscle stimulation was similar between Ctrl and PiC Kd fibers (**Fig 5A, B, G, H**). In response to single twitch stimulation, cytoplasmic Ca^{2+} concentration $[\text{Ca}^{2+}]_c$ increased sharply then decayed rapidly; the amplitude and rate of rise were similar to what we have recorded previously in FDB fibers (15). The peak $[\text{Ca}^{2+}]_c$ and rate of rise of $[\text{Ca}^{2+}]_c$ were similar between Ctrl and PiC Kd fibers (**Fig 5A, C, D**). Decay rate of $[\text{Ca}^{2+}]_c$ was slightly but significantly faster in PiC Kd fibers (**Fig 5E, F**). Compared to single twitch stimulation, tetanic stimulation generated a larger amplitude rise in $[\text{Ca}^{2+}]_c$ that was longer lasting (**Fig 5G**). The amplitude of the $[\text{Ca}^{2+}]_c$ rise was substantially lower in PiC Kd fibers throughout the tetani (**Fig 5G, I, J**). Thus the cytoplasmic Ca^{2+} response to a brief muscle contraction may be largely independent of major Pi transport into mitochondria including its effect on mitochondrial Ca^{2+} uptake. In contrast, the cytoplasmic Ca^{2+} response elicited by longer lasting contraction would be influenced by PiC-mediated Pi transport into mitochondria. It is also possible that PiC Kd fibers have less Ca^{2+} stored in the sarcoplasmic reticulum; this will be tested by measuring $[\text{Ca}^{2+}]_c$ after discharging sarcoplasmic reticulum Ca^{2+} stores using Thapsigargin to inhibit sarcoplasmic reticulum Ca^{2+} uptake via the SERCA pump.

Because muscle force development depends on cytoplasmic Ca^{2+} , we evaluated skeletal muscle force generation *ex vivo* in isolated Extensor digitorum longus (EDL) muscle (16) using similar

single twitch and tetanic stimulation protocols as used to investigate $[Ca^{2+}]_c$ in FDB fibers. Specific force (force, N, per cross-sectional area of muscle, estimate by EDL weight after the physiology experiment, and was unchanged in PiC Kd EDL (**Fig 6A**)) was decreased (faster) in PiC Kd EDL in response to both stimulation protocols (**Fig 6B, D**). The time-to-peak was also decreased during PiC Kd EDL in response to single twitch stimulation (**Fig 6C**). The largest change in PiC Kd EDL was an almost doubling (slowing) of the time-to-peak in PiC Kd EDL challenged with tetanic stimulation (**Fig 6E**). These results are consistent with slower cross-bridge cycling due to lower cytosolic $[Ca^{2+}]$. They could also reflect a deficit in ATP, due to a lesser oxidative phosphorylation (related to a limitation in Pi availability) or to a limitation in glycolytically derived ATP.

To further evaluate muscle performance, the kinetics of muscle force loss during a fatigue protocol and force recovery from fatigue were measured. We reasoned that, if ATP were limiting in PiC Kd EDL, then PiC Kd muscle would fatigue faster. However, this was not the case because the PiC Kd EDL maintained force for longer during the fatigue protocol (**Fig 6F, G**). Moreover, muscle force recovered faster in PiC Kd EDL (**Fig 6H, I**). These observations argue against a limitation in ATP as an explanation for the different force generation characteristics observed in PiC Kd EDL.

Preliminary Discussion:

The mouse model of inducible skeletal muscle-specific loss of PiC provided an opportunity to revisit, in a genetic, classic questions about the role of mitochondrial Pi transport and mitochondrial Ca^{2+} handling. Importantly, the genetic mouse model allows tissue-level Ca^{2+} signaling, including in response to stimulation, to be evaluated, along with the relevant physiology.

Near absence of the PiC did not lower mitochondrial Ca^{2+} uptake, as would have been predicted (7), but rather was associated with a slight increase in the amount of Ca^{2+} taken up into mitochondria. The greater uptake is likely the result of a small increase in expression of the mitochondrial Ca^{2+} uniporter pore forming subunit, MCU, and thus an expansion of the capacity for mitochondrial Ca^{2+} uptake. That mitochondrial Ca^{2+} uptake was not as clearly increased in PiC Kd mitochondria energized with succinate as with malate/pyruvate is consistent with a earlier report showing a Pi-dependence of mitochondrial Ca^{2+} uptake when succinate was used as the substrate (17); in PiC Kd mitochondria energized with succinate, the increase in uptake capacity due to elevated MCU expression may have offset by a slowing of the uptake due to limited Pi transport. Overall, however, the lack of a clear decrease in mitochondrial Ca^{2+} uptake in PiC Kd mitochondria is unlikely to be explained by Pi transport via other transporters, such as the dicarboxylate or ATP-Mg²⁺-Pi transporters since these are expressed only at very low levels in skm mitochondria, both Ctrl and PiC depleted (not shown). Rather, the low residual levels of

PiC in PiC Kd mitochondria, combined with a small increase in uniporter capacity, may be sufficient to support normal or slightly elevated total mitochondrial Ca^{2+} uptake in PiC Kd mitochondria. Indeed, the turnover rate of the PiC was estimated to be 50,000 per second at 25°C (18).

PiC depletion was associated with elevated matrix free Ca^{2+} in response to a Ca^{2+} pulse, even when mitochondrial Ca^{2+} uptake was slowed to be approximately equal to the rate in Ctrl mitochondria. Thus, in the near absence of PiC, and thus of a substantial provisioning of Pi to the matrix, matrix free Ca^{2+} is higher for a given amount of Ca^{2+} taken up, and most likely reflects the absence of formation of calcium-phosphorus precipitates. The existence of these precipitates within mitochondria was long predicted (5), then eventually measured (6) and imaged (8). Yet, it is interesting that, while the limited Pi available in PiC Kd mitochondria is sufficient to support robust mitochondrial Ca^{2+} uptake, it was insufficient to prevent a rise in matrix free Ca^{2+} . Thus, the ability of Pi to sequester Ca^{2+} calcium in the matrix has a greater reliance on the PiC than does mitochondrial Ca^{2+} uptake.

Mitochondrial Ca^{2+} uptake was associated with membrane potential depolarization that was particularly pronounced in PiC depleted mitochondria. Experiments ruled out PTP opening which actually showed a lesser likelihood of opening when PiC was depleted, consistent observations in PiC-depleted heart mitochondria (11) and in support of Pi as a positive regulator of the PTP (10). Studies with oligomycin revealed the pronounced depolarization to be largely oligomycin-sensitive, suggesting that the depolarization reflects a greater stimulation of oxidative phosphorylation (and associated depolarization) in PiC-depleted mitochondria. That oxidative phosphorylation is possible was already evident by the slight hyperpolarization that occurred even in PiC-depleted mitochondria after oligomycin was added. We suggest that the higher free Ca^{2+} in the matrix of PiC-depleted mitochondria was sufficient to stimulate oxidative phosphorylation more in those mitochondria than in mitochondria from control mice; in other words, Ca^{2+} sequestration in calcium-phosphorus precipitates may be capable of limiting the amount of free Ca^{2+} available to stimulate Ca^{2+} -sensitive dehydrogenases and PTP1.

Although the expected decrease in mitochondrial Ca^{2+} uptake was not apparent in PiC-depleted mitochondria, mitochondrial Ca^{2+} uptake and matrix free Ca^{2+} were both altered (increased). Because of the known cross-talk between mitochondrial Ca^{2+} handling and cytosolic Ca^{2+} signals, it was of interest to determine if cytoplasmic Ca^{2+} responses were changed, which was pursued in intact muscle fibers. A single pulse elicited a small cytosolic Ca^{2+} rise that was unaffected by the loss of PiC. The indifference to PiC expression can reflect either that this small Ca^{2+} elevation was insufficient to be transferred to the mitochondria and thus to be influenced by changes in mitochondrial Ca^{2+} handling, or that any changes occurring in PiC-depleted mitochondria did not perturb the cytoplasmic Ca^{2+} response. Compared to response to a single pulse, high frequency stimulation elicited a cytosolic Ca^{2+} response in control fibers that was

higher in amplitude (3x) and longer lasting, and slowly rose during the stimulation. The peak Ca^{2+} elevation in the PiC-depleted fibers was ~30% lower, though the kinetic of the response was the same as in the control fibers. That PiC-depleted fibers showed a similar incrementing pattern of cytosolic Ca^{2+} during the stimulus train indicates that the lower cytosolic Ca^{2+} at the onset of the train is unlikely to reflect lower sarcoplasmic reticulum calcium stores. It should be noted that transcript levels of ryanodine receptor subtype I and SERCA2 were similar between control and PiC Kd muscle, as was transcript levels of parvalbumin (not shown); thus changes in expression sarcoplasmic reticulum Ca^{2+} release and uptake mechanisms, or cytosolic Ca^{2+} binding proteins is unlikely to explain the lower cytoplasmic levels during high frequency stimulation in PiC-depleted fibers. Rather, we suggest that this lower level reflects a greater mitochondrial Ca^{2+} uptake, as suggested by the experiments in isolated mitochondria.

The above observations demonstrate that PiC depletion can substantially alter Ca^{2+} signals in muscle. To gain insight into whether these altered Ca^{2+} signals could translate into altered muscle function, force generation was tested in intact EDL muscle studied *ex vivo* with similar stimuli as was used to evaluate cytoplasmic Ca^{2+} responses in intact fibers. We first note that EDL weights and calculated cross-sectional areas (CSA) were similar in both genotypes. Nonetheless, force was reported relative to CSA since CSA area is a surrogate for contractile protein expression which is a major determinant of the force that an entire muscle can generate. It also seems unlikely that an ATP limitation would explain any differences in PiC Kd muscle, because PiC EDL was more resistant to fatigue and even recovered a little faster than normal muscle. The lower peak force in response to the twitch stimulus in PiC EDL is unlikely to be related to changes in Ca^{2+} because the cytosolic Ca^{2+} response to the single twitch was unaffected by PiC depletion. A substantial difference in the PiC-depleted muscle was a near-doubling in the time taken to attain peak force, and this was in response to the high frequency stimulation that was associated with a profound suppression in the peak Ca^{2+} response; thus it is possible that the lower Ca^{2+} availability translated into a delay in Ca^{2+} delivery to some contractile units, leading to a delay at the level of the whole muscle in the ability to attain a maximal force.

In conclusion, while this study demonstrates that even very low PiC capacity is sufficient to maintain normal mitochondrial Ca^{2+} uptake, greater PiC capacity is needed to provide enough Pi to the mitochondrial matrix to bind to Ca^{2+} to an extent that is seen normally. Yet, the resulting elevation in matrix free Ca^{2+} may allow for greater Ca^{2+} -induced stimulation of oxidative phosphorylation, and, indeed, our observations suggest that Pi buffering of matrix Ca^{2+} might limit the Ca^{2+} available to stimulate oxidative phosphorylation. Our observation in intact fibers and whole muscle suggest that PiC-dependent changes in mitochondrial Ca^{2+} handling indeed impact muscle function.

Aim 3

Aim 3: To generate TAT fusions proteins of PiC and to test if TAT-PiC can be delivered to cells.

In this research we are testing the approach of Protein Replacement Therapy for the treatment of mitochondrial disorders, namely the delivery of a wild type mitochondrial protein directly to its sub-cellular location, in the form of a fusion protein. We are focusing on the mitochondrial PiC carrier, which is localized to the mitochondrial inner membrane.

Humans (and mice (1)) have a documented alternatively spliced exon 3A and 3B, generating two mPiC isoforms, A and B. Thus, we designed and constructed both TAT-MTS-PiCA and TAT-MTS-PiCB *human* isoforms. The natural mitochondrial targeting sequence (MTS) of PiC (49 aa) was used.

As previously reported, TAT-MTS-mPiCA was expressed and calibration experiments were performed to find the bacterial host and conditions for the expression of TAT-MTS-mPiCA fusion protein as well its partial purification. Furthermore, we demonstrated that:

- 1) Partially purified TAT-MTS-PiCA fusion protein is not cytotoxic to cultured cells
- 2) TAT-MTS-mPiCA fusion protein is internalized into cells
- 3) TAT-MTS-mPiCA added to cells correctly localizes to the mitochondrial inner membrane.

In following experiments, we tested the ability of TAT-MTS-mPiCA to improve mitochondrial functionality of cells with decreased expression of mPiC. As mPiC is an essential protein for cells, we could not generate a knock out cell line by the CRISP/Cas9 technology; thus, we used specific si-mPiC for transiently lowering the expression of mPiC.

Transient transfection with esi-mPiC of HeLa cells lowered mPiC-mRNA levels by more than 85%, as determined by qPCR (**Fig 7**). Transfected cells, at 48hr post transfection, grew slower; showing a 43% reduction in cell number (**Fig 8A, B**). Treatment with TAT-MTS-mPiCA fusion protein for 24hr increased cell growth, resulting in ~15% higher cell numbers at the end of the incubation period (**Fig 8A, B**).

We next tested the effect of TAT-MTS-mPiCA fusion protein on mitochondrial activity in HeLa cells with lowered expression of mPiC (treated with si-mPiC) using bioenergetics analyses (**Fig 9A**: using Seahorse technology). Treatment with TAT-MTS-mPiCA fusion protein significantly improved mitochondrial bioenergetics as measured by increased O₂ consumption rate (OCR), showing a 3Xfold increase with 10 µg of fusion protein and 9.3Xfold increase following treatment

with 20 µg of the fusion protein. Treatment with the fusion protein also increased ATP production; 1.8x and 3.3x fold with 10 µg and 20 µg of the fusion protein respectively (**Fig 9B, C**).

These results also demonstrate that improvement of mitochondrial bioenergetics is dose dependent (**Fig 9B, C**). These analyses were repeated 4 times, with similar results.

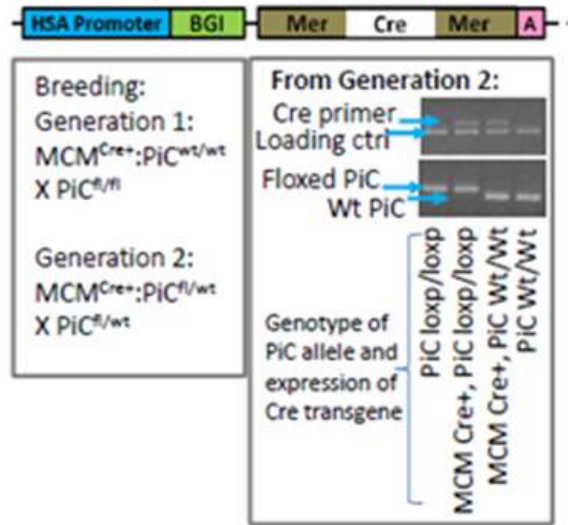
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FIGURE 1

A Mouse model: Tamoxifen-inducible
PiC depletion in skeletal muscle



B Immunoblot:
Skeletal muscle lysate

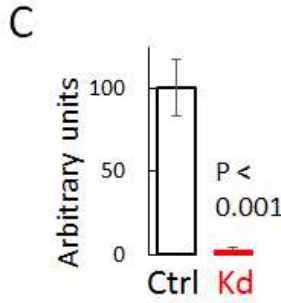


Figure 1. Mouse model of inducible skeletal muscle-specific PiC depletion.

A. Mouse model design and breeding strategy to generate experimental mice with Tamoxifen-induced skeletal muscle-specific PiC depletion, using the Human Skeletal Actin (HSA) Cre recombinase driver that is expressed only in skeletal muscle.

B. Example of immunoblot of PiC in lysates from quadriceps muscle. Grb2 is the loading control. Ctrl: mice with floxed PiC alleles but not expressing HSA-Cre (see Panel A). PiC Kd (“Kd”): PiC with floxed PiC alleles and expressing HSA-Cre (see Panel A). Both Ctrl and PiC Kd mice were treated with Tamoxifen (30 mg/kg, intraperitoneally, 5 consecutive days; tissue was harvested 14 days after the last injection).

C. Quantification of PiC immunoblot; PiC was expressed relative to Grb2; n = 7/genotype. P value: unpaired t-test.

FIGURE 2

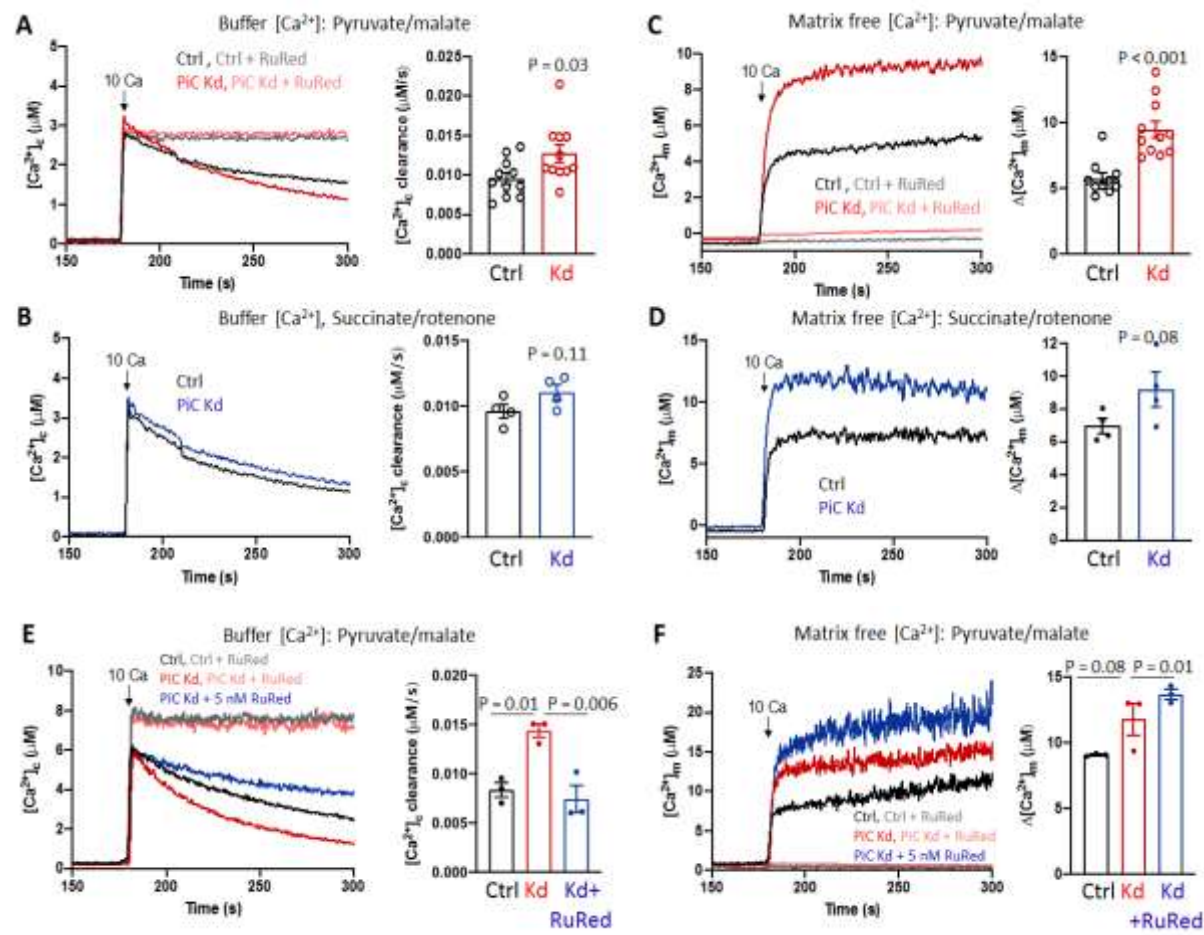


Figure 2. Accelerated mitochondria Ca^{2+} uptake and higher matrix free $[\text{Ca}^{2+}]$ with PiC depletion. $[\text{Ca}^{2+}]$ was measured in mitochondria isolated from skeletal muscle of Ctrl and mice with PiC in skeletal muscle. In all panels, skeletal muscle mitochondria (375 μg) were suspended in an intracellular-like medium, Ruthenium Red (RuRed; 1 μM) was used to inhibit the mitochondrial Ca^{2+} uniporter in some runs, CGP37157 was used to inhibit the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger in all runs and Thapsigargin was added in all runs to inhibit the SERCA pump present on any contamination sarcoplasmic reticulum. “Kd” refers to PiC kd. In all panels, traces are from a representative experiment.

A. Skeletal muscle mitochondria were energized using pyruvate and malate (5 mM each). The $[\text{Ca}^{2+}]$ in the buffer ($[\text{Ca}^{2+}]_c$: “cytosol”) was measured using RhodFF, and calibrated with a Ca^{2+} pulse followed by EGTA at the end of the run. Arrow: addition of 10 μM CaCl_2 . Ca^{2+} clearance ($\mu\text{M}/\text{s}$) was measured as the decrease in the RhodFF signal 30 seconds from the CaCl_2 pulse, relative to the peak. Bar chart and error bars: mean ($\pm$ standard error of the mean (sem)) of individual experiments ($n = 8$ mice/genotype). P value: unpaired t-test.

B. $[\text{Ca}^{2+}]_c$ measured as in A., except skeletal muscle mitochondria were energized using succinate (10 mM) and rotenone (1 μM) was included to inhibit Complex I so as to prevent electron backflow from succinate dehydrogenase and generation of superoxide at Complex I. Arrow: addition of 10 μM CaCl_2 . Bar chart and error bars: mean ($\pm$ sem) of individual experiments ($n = 4$ mice/genotype). P value: unpaired t-test.

C. Matrix free $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_m$) was measured using FURA-AM in skeletal muscle mitochondria energized with pyruvate and malate. Arrow: addition of 10 μM CaCl_2 . $[\text{Ca}^{2+}]_m$ was measured as the increase in fluorescence 30 seconds from CaCl_2 addition, relative to the pre-calcium baseline. Bar chart and error bars: mean ($\pm$ sem) of individual experiments ($n = 8$ mice/genotype). P value: unpaired t-test.

D. $[\text{Ca}^{2+}]_m$ measured as in C., except skeletal muscle mitochondria were energized with succinate (+ rotenone). Arrow: addition of 10 μM CaCl_2 . Bar chart and error bars: mean ($\pm$ sem) of individual experiments ($n = 4$ mice/genotype). P value: unpaired t-test.

E. $[\text{Ca}^{2+}]_c$ measured as in A., in mitochondria energized with pyruvate and malate. Some runs using PiC Kd mitochondria were performed in the presence of a low concentration of RuRed (5 nM) to approximate the mitochondrial Ca^{2+} uptake observed in Ctrl mitochondria. Bar chart and error bars: mean ($\pm$ sem) of individual experiments ($n = 3$ mice/genotype). P value: unpaired t-test.

F. $[\text{Ca}^{2+}]_m$ measured as in C., in mitochondria energized with pyruvate and malate. Some runs using PiC Kd mitochondria were performed in the presence of a low concentration of RuRed (5 nM) to approximate the mitochondrial Ca^{2+} uptake observed in Ctrl mitochondria. Bar chart and error bars: mean ($\pm$ sem) of individual experiments ($n = 3$ mice/genotype). Statistics: Analysis of variance (repeated measures) and Tukey post-hoc tests (P values).

FIGURE 3

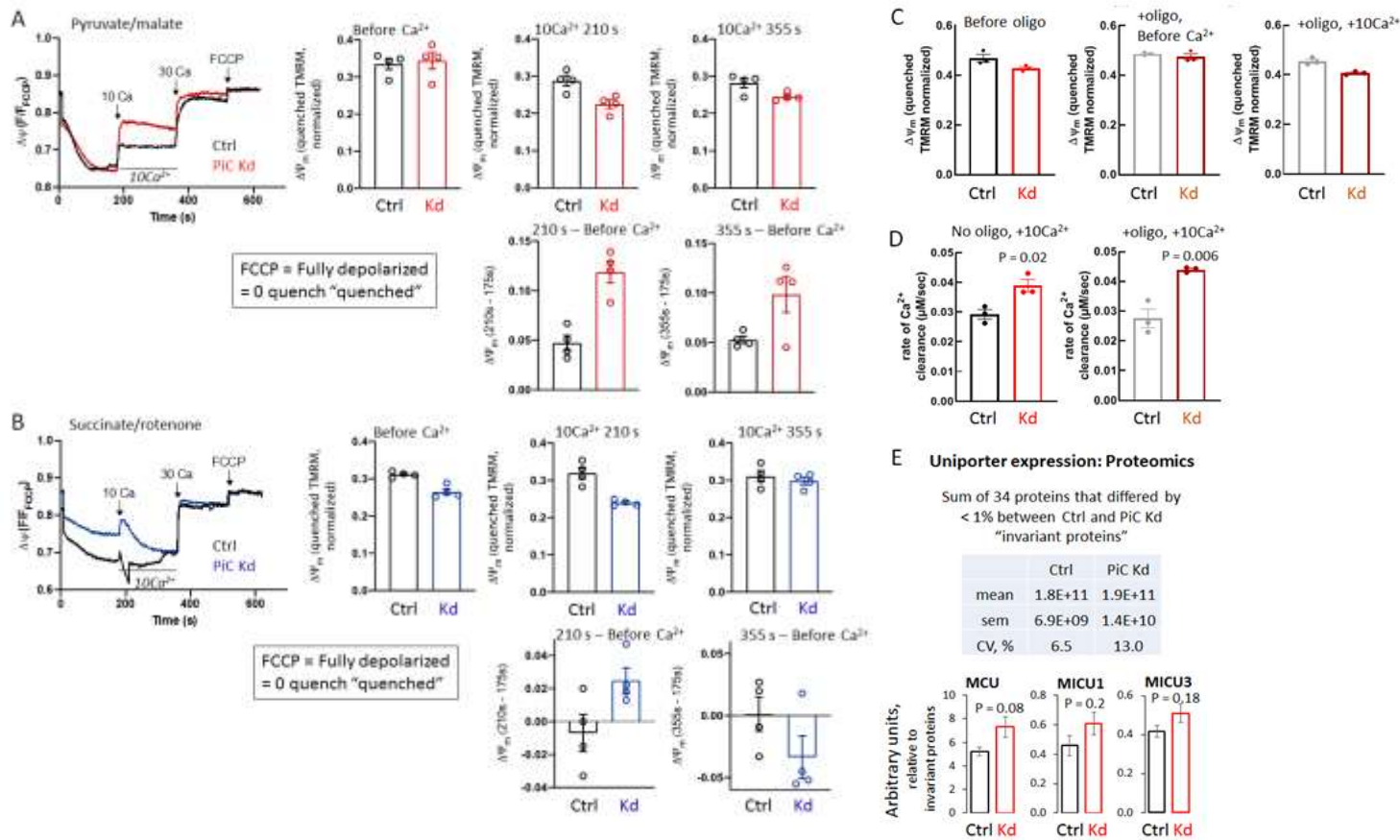


Figure 3. PiC depletion leads to greater Ca^{2+} -induced oligomycin-sensitive membrane potential depolarization. The membrane potential across the inner mitochondrial membrane ($\Delta\Psi_m$) was measured using TMRM (50 nM) in the quench mode; hyperpolarization quenches the fluorescence, thus a decrease in fluorescence corresponds to membrane polarization and an increase reflects depolarization. The chemical uncoupler FCCP was added to fully depolarize $\Delta\Psi_m$. All measurements: isolated skeletal muscle mitochondria (375 μg). Arrows: CaCl_2 addition (10 μM and 30 μM ; only the 10 μM CaCl_2 response was analyzed), and FCCP (1 μM). “Kd” refers to PiC kd.

A. $\Delta\Psi_m$ was measured in mitochondria energized with pyruvate and malate (5 mM each). Measurements were made prior to CaCl_2 addition, then 30 s after 10 μM CaCl_2 addition (“210 s of the recording”), then again 175 s after 10 μM CaCl_2 addition (“355 s of the recording”). Bar chart and error bars: mean ($\pm$ sem) of individual experiments (n = 8 mice/genotype). P value: unpaired t-test.

B. $\Delta\Psi_m$ was measured in mitochondria energized with succinate (10 mM) and rotenone (1 μM) was also added to prevent electron backflow through Complex I. Measurement timing was as described in Panel A. Bar chart and error bars: mean ($\pm$ sem) of individual experiments (n = 4 mice/genotype). P value: unpaired t-test.

C. $\Delta\Psi_m$ was measured in mitochondria energized with pyruvate and malate (5 mM each). Oligomycin (2.5 $\mu\text{g}/\text{ml}$) was used to inhibit the ATP synthase, thereby inhibiting oxidative phosphorylation and ATP hydrolysis; oligomycin was added prior to addition of 10 μM CaCl_2 . Bar chart and error bars: mean ($\pm$ sem) of individual experiments (n = 3 mice/genotype). P value: unpaired t-test.

D. $[\text{Ca}^{2+}]_c$ was measured (see Figure 1A) under the conditions described in Panel C. Bar chart and error bars: mean ($\pm$ sem) of individual experiments (n = 3 mice/genotype). P value: unpaired t-test, n = 3 mice/genotype.

E. Proteomics using mass spectroscopy was performed on isolated skeletal muscle mitochondria. Shown are values for mitochondrial Ca^{2+} uniporter components: MCU is the pore-forming subunit, MICU1 and MICU3 are regulators. Numbers are values normalized to the average of 34 highly expressed proteins that did not differ between Ctrl and PiC Kd by more than 1% (Table; CV: coefficient of variation)). In all cases, number of peptides per sample was > 3. Bar chart and error bars: mean ($\pm$ sem) of individual experiments. P value: unpaired t-test, n = 3 mice/genotype.

FIGURE 4

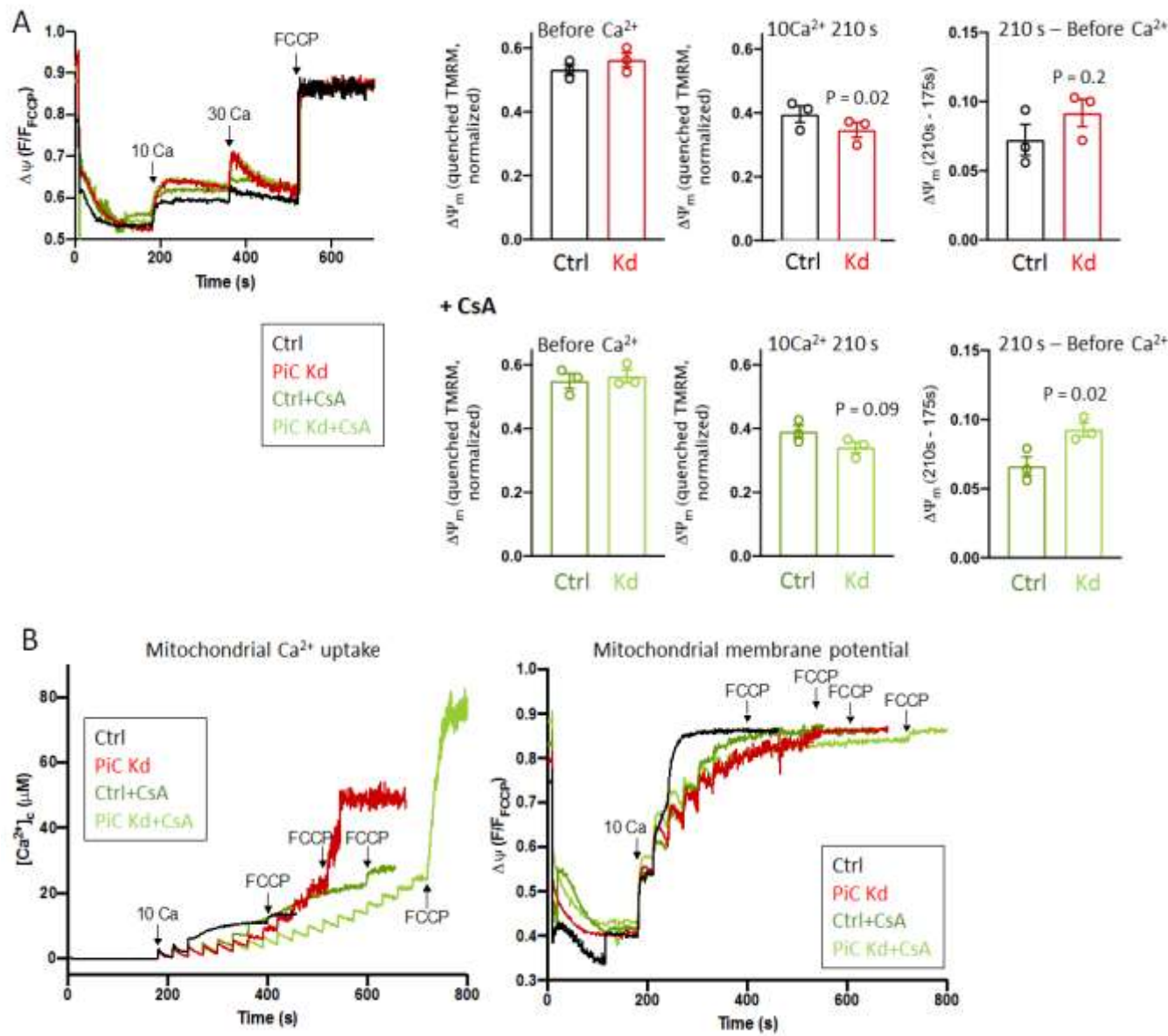


FIGURE 4

Figure 4. Permeability transition pore opening does not account for larger Ca^{2+} -induced depolarization in PiC depleted mitochondria. Experiments were performed in isolated skeletal muscle mitochondria (375 μg). Cyclosporin A (CsA: 1 μM) was used to suppress permeability transition pore opening. The chemical uncoupler FCCP was added to fully depolarize $\Delta\Psi_{\text{m}}$.

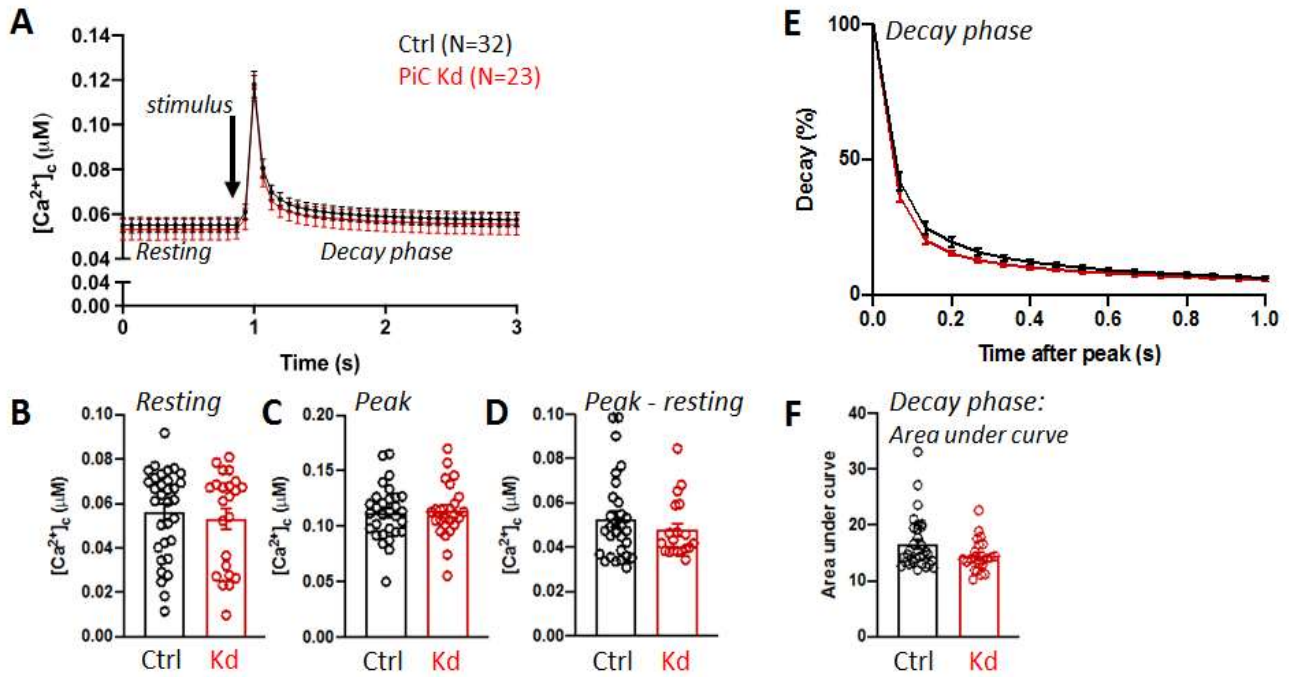
“Kd” refers to PiC kd. In all panels, traces are from a representative experiment.

A. $\Delta\Psi_{\text{m}}$ was measured in mitochondria energized with pyruvate and malate (5 mM each) as in Fig 3A, in the absence (top graphs) and presence (lower graphs) of CsA. Arrows: addition of CaCl_2 (10 and 30 μM , only the 10 μM was analyzed), and FCCP. Bar chart and error bars: mean ($\pm$ sem) of individual experiments, $n = 3$ mice/genotype. Traces are from a representative experiment.

B. Pulses of 10 μM CaCl_2 were added (starting at the arrow, then at each change in $[\text{Ca}^{2+}]_{\text{c}}$) to elicit permeability transition pore opening, indicated by failure of Ca^{2+} uptake (seen as lack of a decrease in $[\text{Ca}^{2+}]_{\text{c}}$) and $\Delta\Psi_{\text{m}}$ depolarization, both of which were suppressed by CsA. Traces are from one experiment.

FIGURE 5

Single twitch stimulus: Panels A - F



Repetitive Tetanic stimulus: Panels G - J

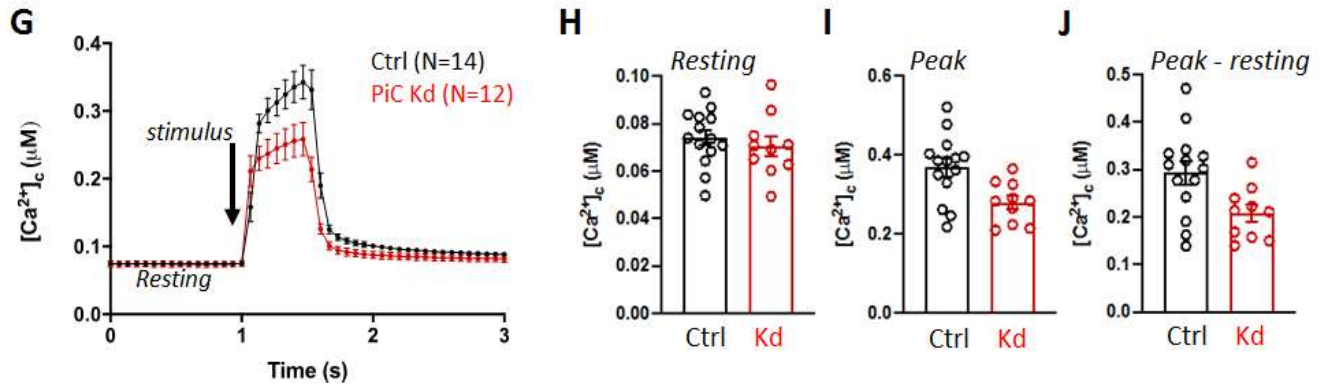


Figure 5. PiC depletion leads to suppression of the cytoplasmic Ca^{2+} elevation in response to tetanic stimulation of muscle fibers. Fibers were isolated from the Flexor digitorum brevis muscle of the foot and attached to coverslips, then loaded with FURA2-AM (2 μM) to measure cytoplasmic $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_c$). Recordings were made in an extracellular-like medium, with glucose as the substrate, and in the presence of N-benzyl-p-toluene sulphonamide (BTS, type II actomyosin inhibitor; 75 μM) to inhibit muscle contraction. “Kd” refers to PiC kd. In all panels, traces are from a representative experiment, P values are from unpaired t-test, n = 7 mice/genotype.

- A.** $[\text{Ca}^{2+}]_c$ was measured in response to a single twitch stimulation (2 V, 2 ms pulse length). Values are mean ($\pm$ sem) from 32 Ctrl and 23 PiC Kd fibers.
- B.** Quantification of “resting” $[\text{Ca}^{2+}]_c$ prior to single twitch stimulation. Bar chart: mean ($\pm$ sem). No significant difference between Ctrl and PiC Kd.
- C.** Quantification of peak $[\text{Ca}^{2+}]_c$ in response to single twitch stimulation. Bar chart: mean ($\pm$ sem). No significant difference between Ctrl and PiC Kd.
- D.** Quantification of the increase in $[\text{Ca}^{2+}]_c$ (peak minus resting) in response to single twitch stimulation. Bar chart: mean ($\pm$ sem). No significant difference between Ctrl and PiC Kd.
- E.** Decay kinetics following single twitch stimulation.
- F.** Quantification of decay kinetics (Panel E). Bar chart: mean ($\pm$ sem) area under the curve.
- G.** $[\text{Ca}^{2+}]_c$ was measured in response to a train of tetanic stimuli (100 tetani, 100 Hz for 500 ms every 2.5 s, pulse length 2 ms, 2 V). Values are mean $\pm$ sem from 14 Ctrl and 12 PiC Kd fibers (from 7 mice/genotype).
- H.** Quantification of resting $[\text{Ca}^{2+}]_c$ prior to tetanic stimuli. Bar chart: mean ($\pm$ sem). No significant difference between Ctrl and PiC Kd.
- I.** Quantification of peak $[\text{Ca}^{2+}]_c$ in response to tetanic stimuli. Bar chart: mean ($\pm$ sem).
- J.** Quantification of the increase in $[\text{Ca}^{2+}]_c$ (peak minus resting) in response to repetitive tetanic stimuli. Bar chart: mean ($\pm$ sem).

FIGURE 6

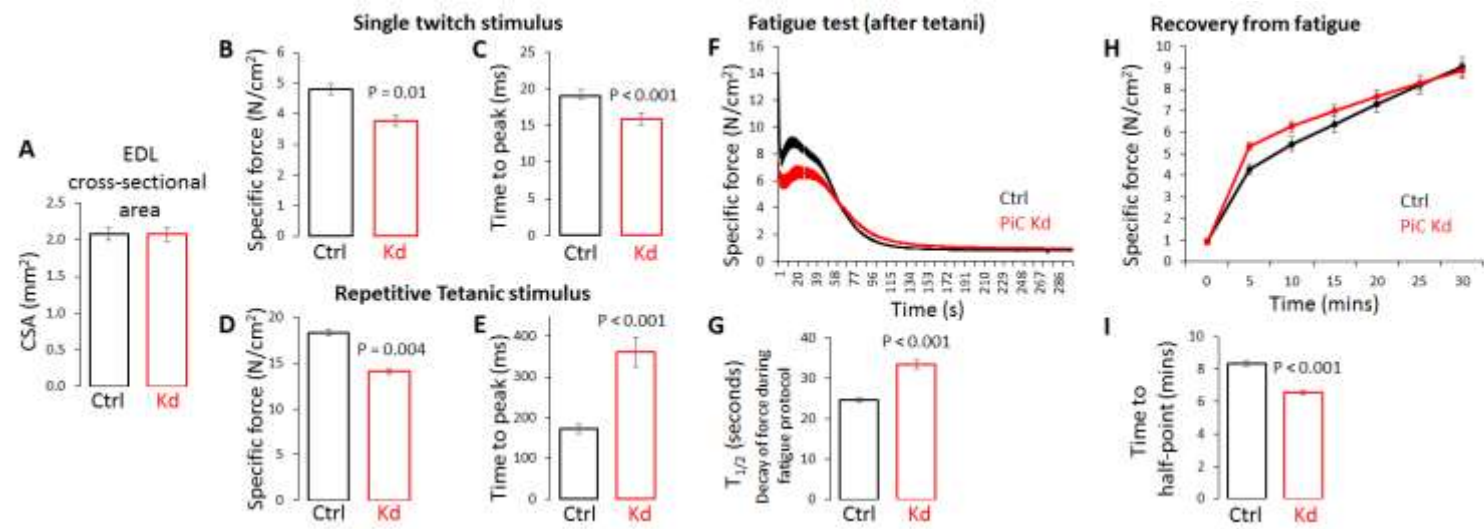


Figure 6. Lower peak force generation and slower force development in response to tetanic stimulation in PiC-depleted muscle. Extensor digitorum longus (EDL) muscle was isolated, suspended and superfused with an extracellular medium containing glucose, then the length at which force is minimum was determined and served as the starting length for subsequent stimulation experiments. In all cases, P values are from unpaired t-test, n = 6 mice/genotype.

A. Cross sectional area of EDL, calculated based on the wet weight of the EDL measured after each experiment. No significant difference between Ctrl and PiC Kd.

B. Maximal force generated during a single twitch stimulus (30 V, 0.2 ms pulse length), normalized to muscle cross-sectional area.

C. Time-to-peak force in response to a single twitch stimulation.

D. Maximal force generated in response to a train of tetanic stimuli (30 V, 120 Hz, 0.2 ms pulse length, each train lasting 500 ms), normalized to muscle cross-sectional area.

E. Time-to-peak force in response to a train of tetanic stimuli.

F. Fatigue test: 5 min after the last maximal tetanic stimulus, muscles were stimulated every second for 8 min with 40-Hz pulses lasting 330 ms. Following the fatigue protocol, a burst of 50 maximal tetanic contractions (120 Hz for 500 ms) was applied to ensure complete fatigue across all samples. Shown are mean ($\pm$ sem) for all samples, during the entire fatigue protocol.

G. Quantification of fatigue by exponential decay fitting to calculate $T_{1/2}$. Bars show the mean ($\pm$ sem) rate of decay, expressed as the $T_{1/2}$, of specific force during the fatigue protocol.

H. Recovery from fatigue test: The recovery protocol started 1 s after the last burst contraction of the fatigue protocol. A maximal tetanic stimulation (120 Hz for 500 ms) was given every 5 min for 30 min. Shown are mean ($\pm$ sem) for all samples, during the entire recovery phase.

I. Quantification of recovery was calculated as the time to reach 50% of the value calculated at 30 mins (last time point of the recovery protocol), based on a logarithmic fit of each recovery time course ($R^2 > 0.95$); bars show the mean ($\pm$ sem).

FIGURE 7

esi-mPiC reduces mPiC mRNA levels in Hela cells

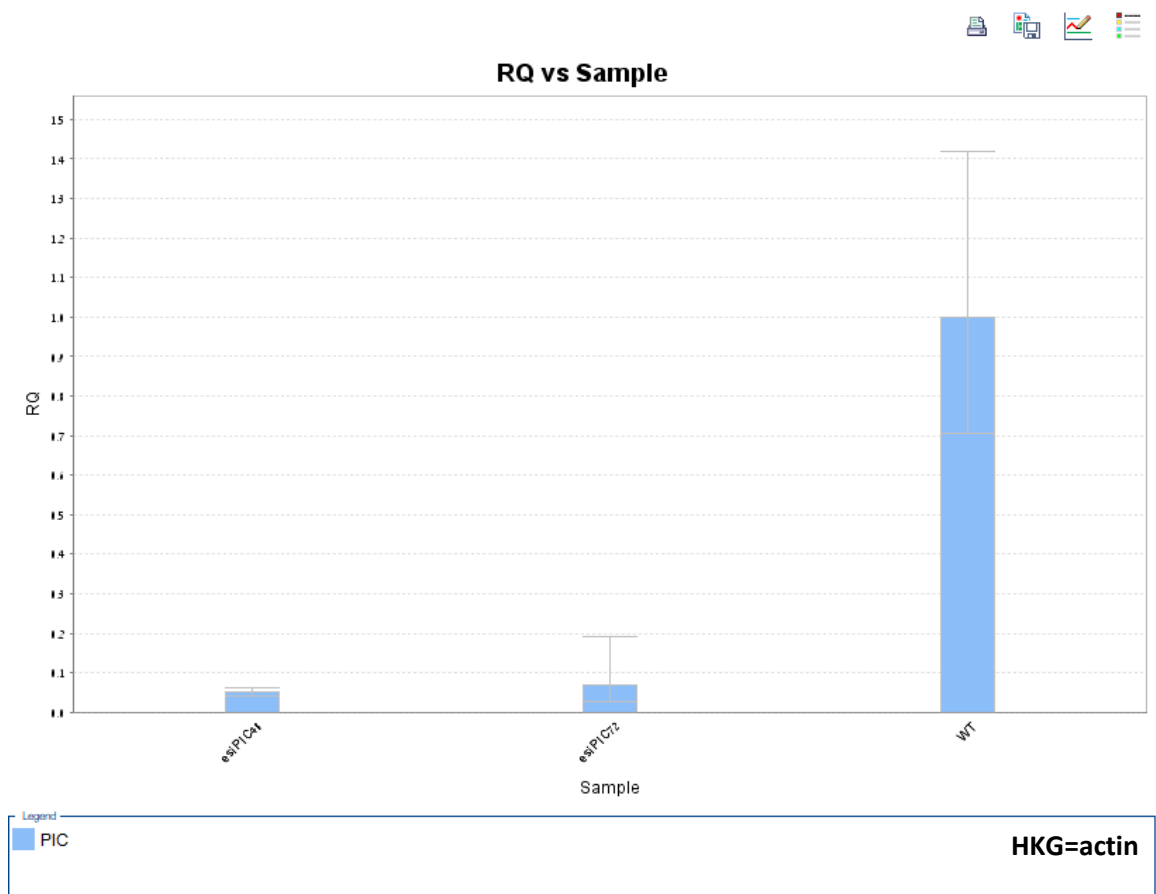


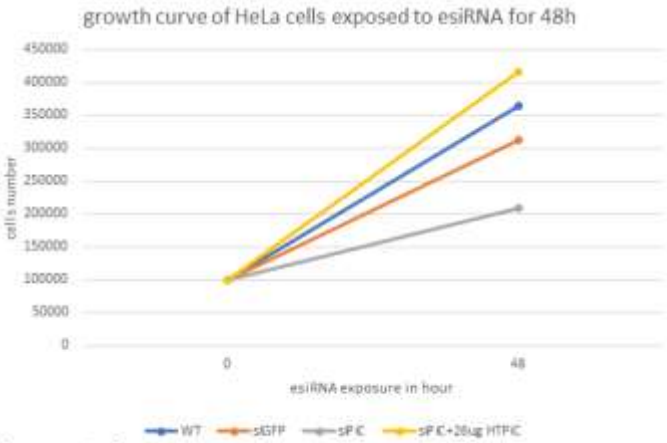
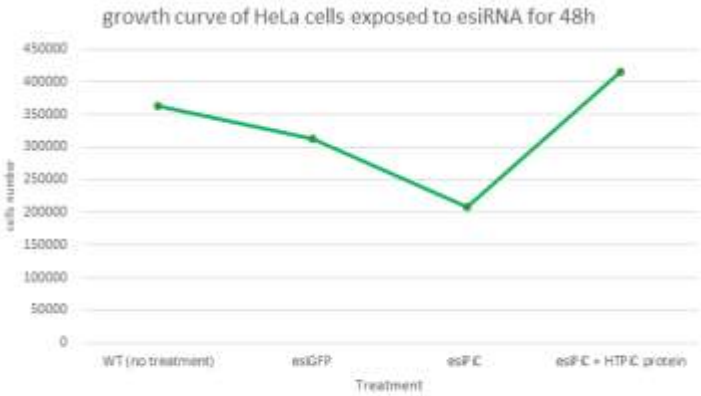
Figure 7. Transient PiC knockdown (esi-mPiC) lowers mPiC mRNA levels in Hela cells. Cells were transfected with esi-mPiC (20 nM final concentration per well). After 24 hrs incubation, the media (rich media) was replaced and cell were incubated for an additional 24 or 48 hrs. RNA was isolated at 48 hrs or 72 hrs post infection and qPCR was performed using specific primers for mPiCA.

FIGURE 8

esi-mPiCA reduces cell growth of Hela cells

Day 1	4x 6 well plates 10 ⁵ cells each
Day 2	esiRNA transfection (WT+esiGFP+2xesiPiC)
Day 3	3x medium replacement with fresh medium for (WT+esiGFP+esiPiC) 1x adding 26ug HTPiC protein 1xsiPiC
Day 4	4x plates cells counting

HeLa cell number	
	48 hours
WT (no treatment)	364000
esiGFP	312000
esiPiC	208000
esiPiC+HTPiC protein	416000



* HTPiC: His-TAT-MTS-PiC: exogenous TAT-MTS-mPiCA fusion protein.

Figure 8. esi-mPiCA slows proliferation rate of Hela cells. Hela cells were transfected with esi-PiCA for 24 hrs. Then, incubation media was changed to rich media and TAT-MTS-mPiC fusion protein was added for an additional 24 hrs, at two concentrations and cell number was determined using trypan blue staining.

FIGURE 3A

Effect of TAT-MTS-mPiCA fusion protein on si-mPiC transfected Hela cells: SeaHorse analysis -Study Design

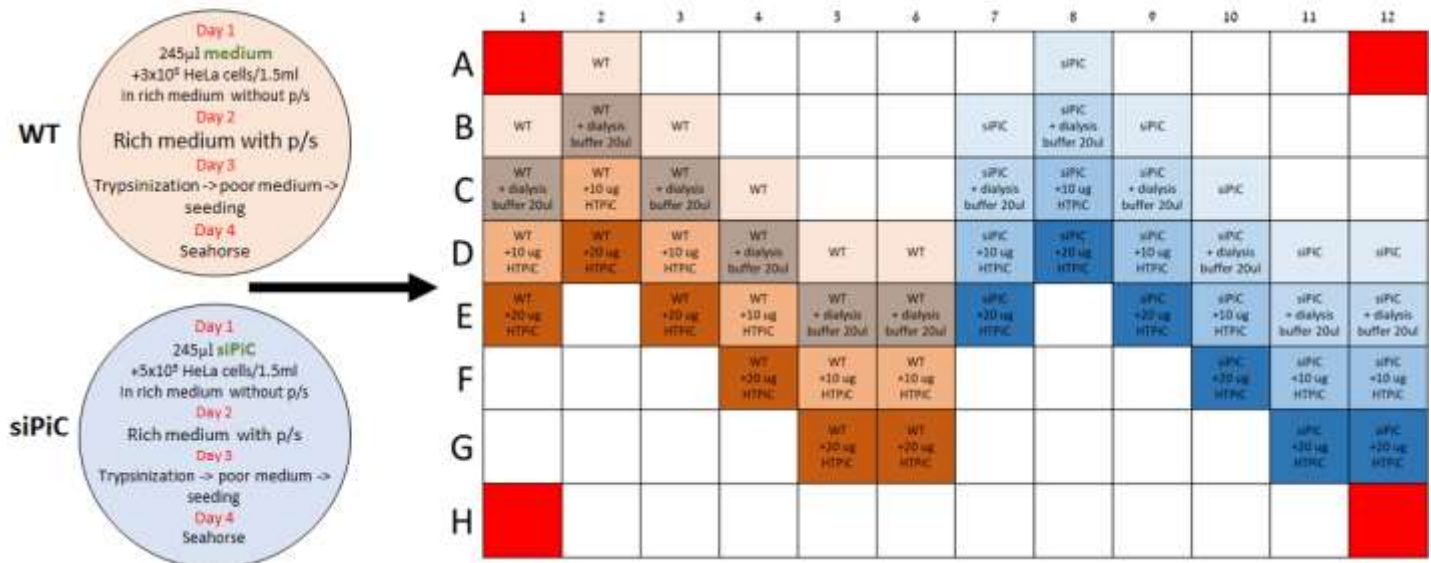


FIGURE 3B

TAT-MTS-mPiC fusion protein improves mitochondrial activity of cells with reduced expression of mPiC

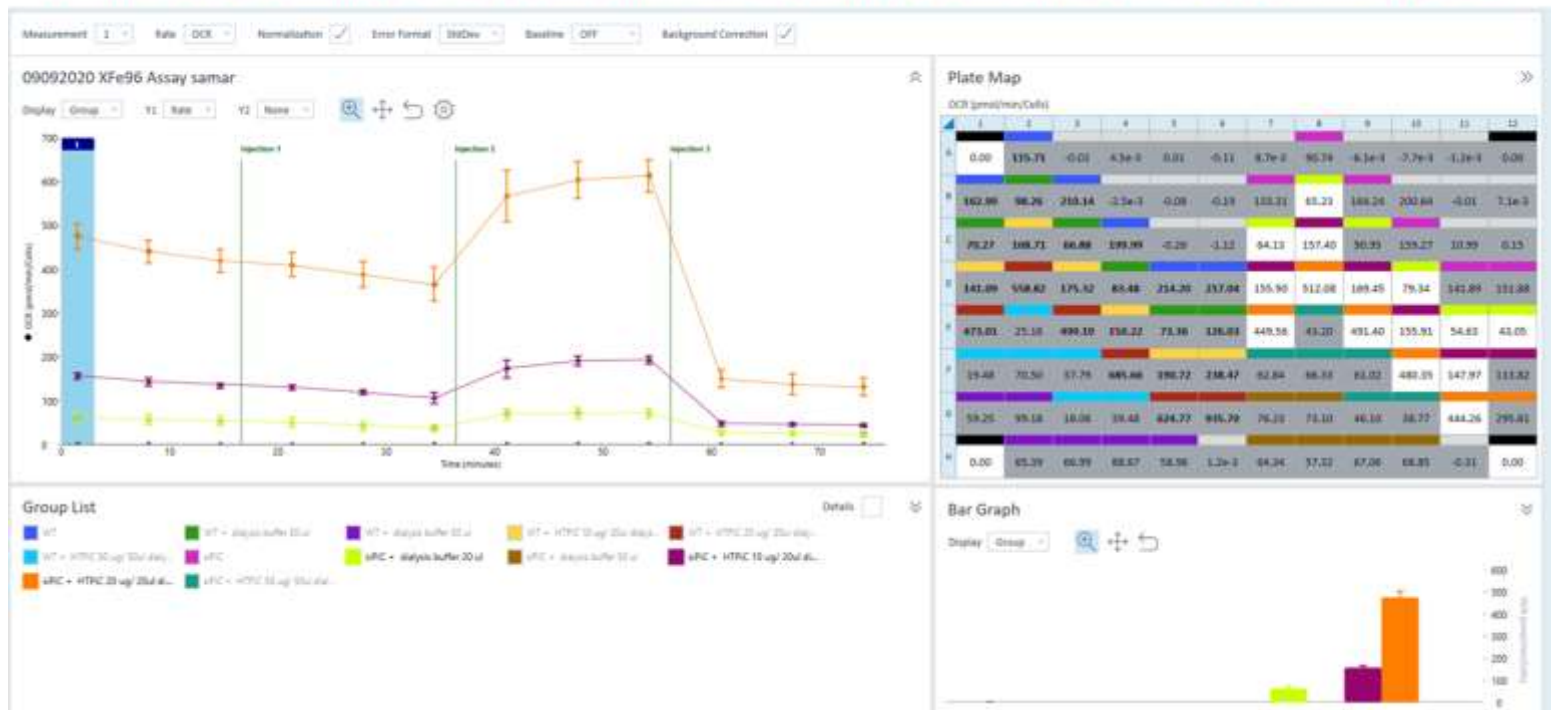


FIGURE 3C

TAT-MTS-mPiC fusion protein improves mitochondrial activity of cells with reduced expression of mPiC

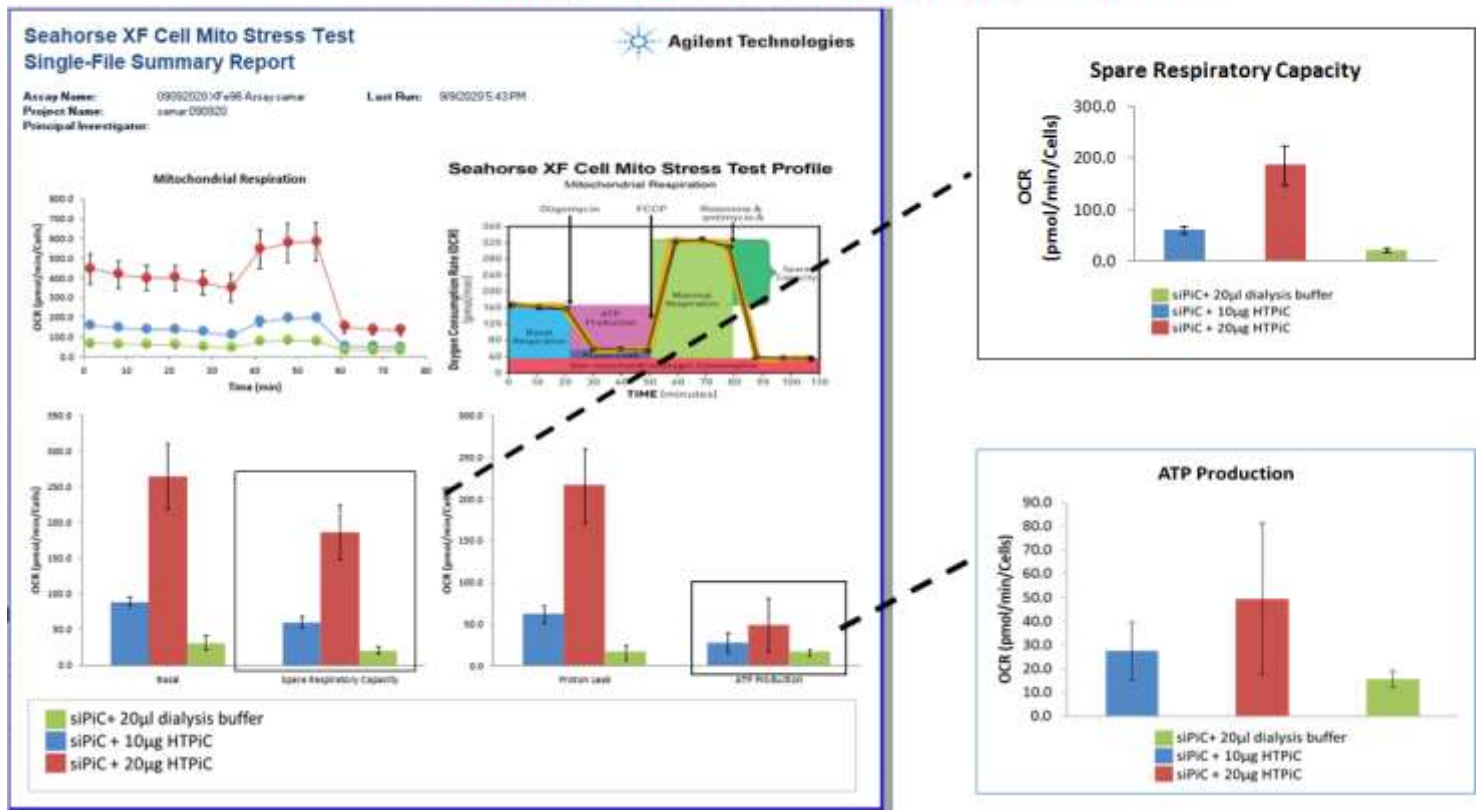


Figure 9. Treatment with TAT-MTS-mPiCA fusion protein improves the mitochondrial bioenergetics of si-mPiC HeLa cells.

A. Study design

B. TAT-MTS-mPiCA fusion protein improves mitochondrial bioenergetics of cells with decreased expression of mPiC. HeLa cells were transfected with esi-mPiCA. After 24 hrs incubation, the media (rich media) was replaced and cells were incubated for an additional 24 hrs. The media was then replaced to poor media without glucose (supplemented with dialyzed FBS, 1% glutamine and 1% p/s). Four hours later TAT-MTS-mPiCA fusion protein was added for an additional 24 hrs, at two concentrations. Mitochondrial function was tested using Seahorse technology.

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?**

-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-PiC 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. 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, and Ms. Samar Zabit (PhD student, Dr. Lorberboum-Galski's lab) in recombinant protein production and purification, and cellular subfractionation.

5. CHANGES/PROBLEMS

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

No changes in the approach

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

Experiments in Dr. Seifert's lab and Dr. Lorberbaum-Galski's lab were suspended from mid-March through to mid-June, due to the worldwide pandemic. Work in both labs has been slow to resume because of the need to maintain a low density of people in the labs, and because it has taken several months for the mouse colony to rebound after most breeding was suspended in mid-March.

- **Changes that had a significant impact on expenditures**

NO CHANGES

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Please see the above paragraph addressing significant delays.

- **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

- **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)**

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. However, this is in the testing phase. 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):	0000-0002-8416-5763
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):	0000-0003-3813-2570
Nearest person month worked:	1
Contribution to Project:	Gyorgy Hajnoczky has performed work in the area of Aim 2 (design of the Ca ²⁺ studies)
Funding Support:	NA

Name:	Cesar Vasquez
Project Role:	Post Doc fellow

Researcher Identifier (e.g. ORCID ID):	0000-0003-2606-6860
Nearest person month worked:	9
Contribution to Project:	Cesar Vasquez has performed work in the area of Aim 2 (Ca ²⁺ flux studies)
Funding Support:	NA

Name:	Heli Xu
Project Role:	Post-Doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	0000-0002-9792-2727
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:	Brianna Hymms
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	2
Contribution to Project:	Brianna 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:	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: expression (both two mPiC isoforms-A&B) in bacterial hosts; purification of the fusion proteins (mainly improving and establishment of a purification protocol), 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, in cultured cells with reduced endogenous expression (by siPiCm) of the PiC mitochondrial carrier.
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: expression (both two mPiC isoforms-A&B) in bacterial hosts; purification of the fusion proteins (mainly improving and establishment of a purification protocol), 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, in cultured cells with reduced endogenous expression (by siPiCm) of the PiC mitochondrial carrier.
Funding Support:	NA

Name:	Tejvir S. Khurana
Project Role:	PI (Univ of Pennsylvania/Consortium)
Researcher Identifier (e.g. ORCID ID):	0000-0003-1337-846X
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:

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 has ended:

Title: Impact of Frataxin deficiency on cardiac substrate metabolism

Role: Principle Investigator

Effort: 0 CM

Agency: Friedreich Ataxia Research Alliance (FARA)

Grants Officer/Address: Jennifer Farmer

Performance Period: 09/01/2016-12/31/19

Level of Funding:

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:

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 level of funding has been updated:

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: 09/01/2017-08/31/2021

Level of Funding:

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 Ca^{2+} , and Ca^{2+} regulated functions.

Overlap: Currently, Aims 1 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 end date has been updated:

Title: Study of the mitochondrial-cellular response to environmental stress by fluorescence 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: 7/14/17-06/30/21* (R33) NCE

Level of Funding: full funding

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: 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:

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 Active Research Support:

The Below Research support/award level of funding has been updated:

Title: Molecular Mechanisms of Mitochondrial Ca²⁺ Transport

Identifier: R01 GM102724

PI: Hajnoczky

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: direct costs for current year

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: Redox regulation of intracellular calcium signaling

Identifier: R01 DK103558

MPI: Hajnoczky & Joseph

Time Commitment: 0.12 Calendar Months

Supporting Agency: NIH/NIDDK

Contracting/Grants Officer: Craig Bagdon, bagdonc@niddk.nih.gov

Performance Period: 4/01/2015 – 3/31/21 NCE

Level of Funding: 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 calendar months and end date has been updated:

Title: Study of the mitochondrial-cellular response to environmental stress by fluorescence imaging

Identifier: R21 ES025672/4R33 ES025672

MPI: Hajnoczky & Csordas

Time Commitment: **0.24 Calendar Months**

Supporting Agency: NIH/NIEHS

Contracting/Grants Officer: Barbara Gittleman, gittlemanbj@niehs.nih.gov

Performance Period: 7/14/17-06/30/21 NCE (R33)

Level of Funding: Hajnoczky direct portion, NCE

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: 0.48 Calendar months

Agency: NIH

Grants Officer/Address: Vernon Anderson, Ph.D.

Performance Period: 09/01/17-08/31/21

Level of Funding: Hajnoczky direct costs

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 Ca^{2+} , and Ca^{2+} regulated functions. Overlap: Currently, Aims 1 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: 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

The Below Research support/award level of funding has been updated:

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: direct costs

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 calendar months and level of funding has been updated:

Title: Targeting Mitochondria To Treat Heart Disease

Hajnoczky PI

Time Commitment: **0.84 Calendar Months**

Supporting Agency: Fondation Leducq

Contracting/Grants Officer: David Tancredi, Executive Director

Performance Period: 04/01/19-12/31/21

Level of Funding: direct costs

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

The Below Research support/award has ended:

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:

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 3×10^{15} 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: 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:

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 has ended:

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:

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:

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 has ended:

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 Yisum

Grants Officer: Mr. Natan Beilinson

Performance Period: 2019-2020

Level of Funding:

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

Not Applicable