Investigating the effect of Calhm2 knockout on oxidative capacity and mitochondrial substrate selection

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Word count (excluding figure legends and acknowledgements): 1408

Background

Mitochondria are essential for energy production, with the complex factors determining its substrate selection and relationship with development being incompletely defined. Notably, the shift from glycolysis to fatty acid oxidation (FAO) accompanies the differentiation and maturation of cardiomyocytes in the developing murine heart. Previous work in the group (unpublished) found that the novel gene *Calhm2* (calcium homeostasis modulator protein 2) was mutated in patients with congenital heart defects. Its protein product CALHM2 has been shown to interact with FAO enzymes. However, whether this interaction is functional, and by extension how CALHM2 is involved in mitochondrial substrate selection, remains unknown. Determining whether a loss of *Calhm2* expression results in reduced FAO capability of cardiomyocytes would provide insight into *Calhm2*'s role in cardiac development. Furthermore, it would underscore the importance of FAO in heart maturation and pathology. CALHM2 may therefore present a novel therapeutic target for the treatment of various heart diseases.

Aims

The overarching goal of this project was to investigate the effect of CALHM2 on FAO capability in mouse embryonic fibroblasts (MEFs) as a model system.
To this end, the aims were:

- Selection and culture of wild-type and *Calhm2*-knockout MEFs, determining *Calhm2* expression levels in the samples using qPCR and Western blotting.
- Metabolic characterisation of wild-type and *Calhm2*-knockout MEFs using specific pathway inhibitors to study substrate catabolism preferences under basal and high-energy-demand states.

The successful achievement of these aims would support the research team's overarching goal of elucidating the role of CALHM2 in regulating cardiac metabolism. The project would also provide the basis for further studies into the specific pathways modified by CALHM2 depletion.

Materials and methods

Cell culture: MEFs were isolated from mouse embryos harvested between days 12.5 to 14.5 of gestation. Five cell lines (three wildtype, two knockout) were then cultured in Dulbecco's

modified Eagle medium (DMEM), high glucose, pyruvate (Cat#41966-029) supplemented with 10% fetal bovine serum (Cat#10500-064), 1x MEM amino acids (Cat#11140-035) and penicillin steptomycin (Cat#15070-063), and 50μM 2-mercaptoethanol (Cat#31350-010). Cells were grown at 37°C in a 5% CO_2 incubator, passaged at 70–90% confluency in T175 flasks, and detached with 0.25% trypsin, phenol red (Cat#25050-014). Experiments were performed between passages 3 and 6. All solutions were sourced from ThermoFisher Scientific.

qRT-PCR: RNA was isolated from cell pellets formed by centrifuging using the ReliaPrep™ RNA Cell Miniprep system, with concentrations assessed using a NanoDrop spectrophotometer. cDNA was retrotranscribed from purified RNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Cat#4368814). SYBR green qPCR was performed using 5μL of SYBR™ Select Master Mix (ThermoFisher Scientific), 1 μL of 10ng/μL forward and reverse primers, and 0.5μL nuclease-free water per well. Each combination of cDNA and primers was assayed in three technical replicates per biological replicate. Expression levels were evaluated using the ΔΔCt method; the expression of Rpm127 and B2m housekeeping genes were used for normalisation.

Western blot: Proteins were solubilised from cell pellets using RIPA lysis buffer (150mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8.0, protease inhibitor). Total protein concentration was determined using BCA assay. Proteins were separated via SDS-PAGE and transfered to a nitrocellulose membrane. Membranes were blocked for one hour at room temperature with rotation with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) supplemented with 0.1% Tween. Overnight incubation at 4°C with rabbit PAb 203542-T32 preceded three washes in TBS-Tween, which was followed by incubation with anti-rabbit horseradish-peroxidase-conjugated secondary antibody. Membranes were scanned using a Licor Odyssey imager.

Genotyping: HotSHOT DNA extraction was performed prior to KOMP PCR genotyping with Floxed, PreCre, and Wildtype primers. PCR products were loaded into a 1-2% agarose gel and imaged after separation.

Seahorse XF-96 energetic analysis: Following the optimisation of cell density and FCCP concentration per well, MEFs from one wildtype and one knockout cell line were plated in at least 4 replicate wells at a density of 15000 cells/well, with each well made up to 200μL growth media in a 96-well Seahorse plate (Agilent, Cat#103774-100). Cells were counted using a hemocytometer. After plating, cells were placed at room temperature under a tissue culture hood for one hour prior to overnight incubation at 37°C in a 5% CO_2 incubator. Growth media was replaced with 180μL Seahorse XF DMEM media (Agilent, Cat#103575-100); the assay-specific addition of glucose, glutamine, and pyruvate to media followed Agilent Mito Stress Test, Mito Fuel

Flex Test, and Real-Time ATP Rate Assay instructions. All plates were normalised to cell count using BCA assay.

Results and outcomes

Characterisation of wildtype and *Calhm2*-knockout MEFs

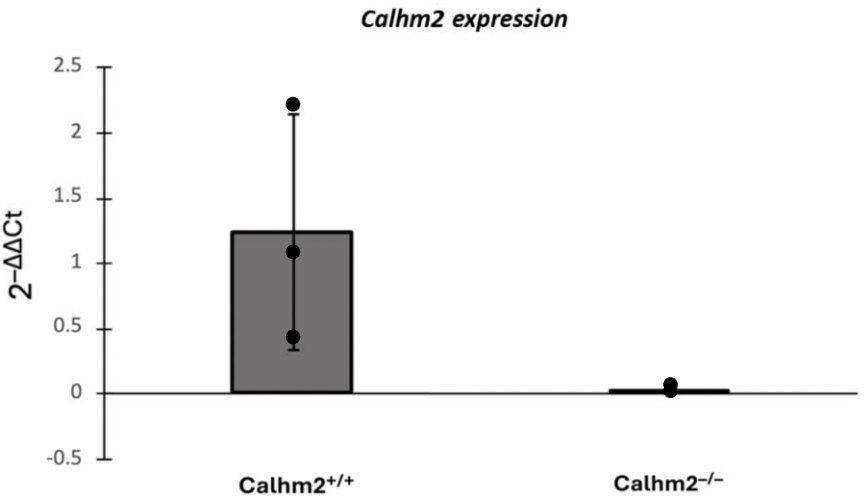

Figure 1. *Calhm2* expression in wildtype (n=3) and knockout (n=2) MEFs determined using qRT-PCR. Error bars represent the standard deviation in $2^{-\Delta\Delta Ct}$ between samples.

To characterise the effect of *Calhm2* knockout on oxidative capacity and substrate selection, the presence or absence of *Calhm2* expression in our MEF cell lines required confirmation. Two successful qRT-PCR assays confirmed the expected genotypes of our MEF cell lines for downstream applications, the latter of which is represented in Figure 1.

Energetic analysis of MEF cell lines

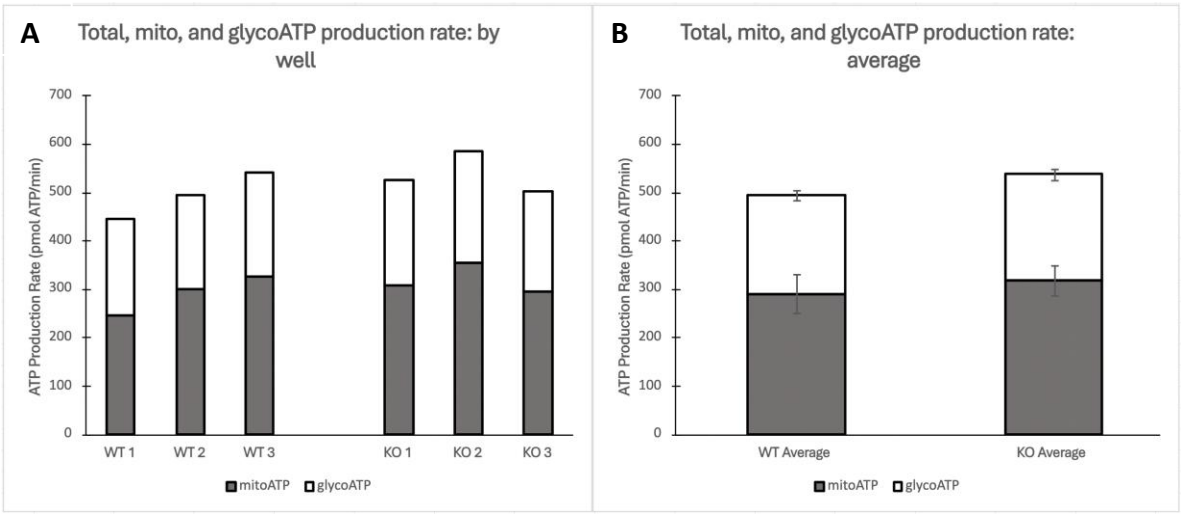

Figure 2. Rates of total cellular ATP, mitoATP, and glycoATP production for wildtype (n=1) and knockout (n=1) MEF cell lines calculated from OCR and extracellular acidification rate (ECAR) data collected using the Real-Time ATP Rate Assay (ATPRA). The ATPRA measured total ATP production rates, permitting the quantification of ATP production from mitochondrial oxidative phosphorylation (OXPHOS, mitoATP) and glycolysis (glycoATP). Total cellular ATP is equal to the sum of mitoATP and glycoATP production rates. Basal OCR and ECAR rates were measured prior to injection of oligomycin, which inhibits mitoATP production. Subsequently, the injection of a mixture of rotenone and antimycin A results in complete inhibition of mitochondrial respiration, removing the effect of CO2-dependent acidification. OXPHOS contributes to the OCR, permitting the rate of ATP production associated with OXPHOS (mitoATP production rate) to be calculated from assay data. Additionally, both OXPHOS and glycolysis contribute to the ECAR: the former through CO2 production by the TCA cycle contributing energy to OXPHOS, the latter through the production of protons accompanying conversion of glucose to lactate. Taking the buffer factor of the assay medium into consideration, total proton efflux rate (PER) was calculated from ECAR data. Following the injection of rotenone-antimycin A, the resultant PER data allowed quantification of the rate of ATP production rate from glycolysis (glycoATP production rate). Measurements were made in triplicate per cell line (Figure 4A) and averages are shown in Figure 4B. Calculations were performed as instructed in the user manual.

Quantifying the proportion of total cellular ATP production that is constituted by mitoATP and glycoATP production is useful for determining whether MEFs are a suitable model system to study the effects of *Calhm2* knockout on metabolism. Previous work in the group suggested that MEFs were primarily glycolytic; if MEFs are mostly glycolytic, then they may not shift to oxidative metabolism regardless of *Calhm2* expression, necessitating the use of a different cell model. To investigate this, we performed an ATPRA, which also contributes to the primary goal of determining how *Calhm2* knockout affects the rate of oxidative metabolism.

Contradicting what was suggested by some prior work, this assay provided evidence that the MEFs have a significant oxidative component and therefore could be a useful cell model for this project (Figure 2). However, the balance between mitoATP and glycoATP production was found to have no significant difference between the wildtype and knockout cell lines; this was investigated further in the below tests.

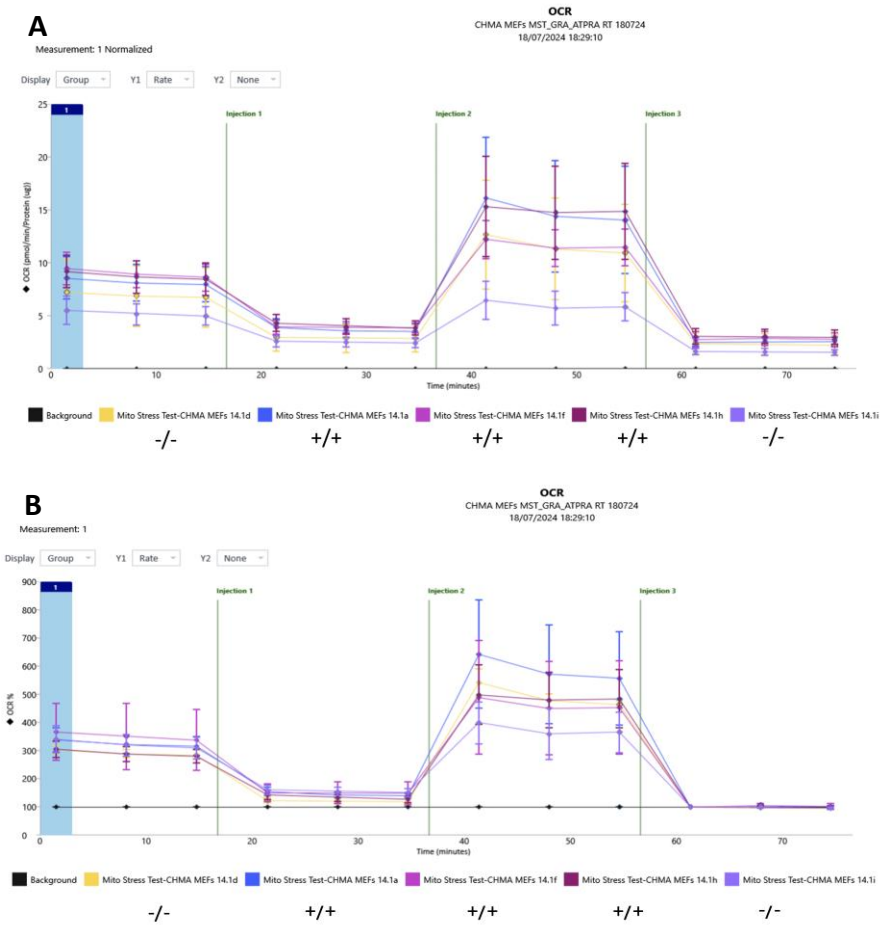

Figure 3. Changes in real-time oxygen consumption rate (OCR) for wildtype (n=3) and knockout (n=2) MEFs in response to modulators of respiration, determined using the Seahorse XF Mito Stress Test. The Mito Stress Test provides an initial characterisation of the baseline glycolytic and oxidative capacity of MEFs. Figure 2A displays data normalised to cell count; Figure 2B displays relative OCR %. Basal respiration is first established and quantified prior to the first injection of oligomycin. Oligomycin inhibits ATP synthase, decreasing electron flow through the electron transport chain (ETC); the resultant drop in OCR represents the proportion of ATP-linked respiration being performed. The uncoupling agent FCCP is injected next and causes uninhibited electron flow through the ETC, leading to a rise in OCR that represents maximal respiration. The difference between basal and maximal respiration is defined as spare respiratory capacity. The third injection, a mixture of rotenone and antimycin A, inhibits complexes I and III respectively: this suppresses mitochondrial respiration, with the remaining OCR corresponding to non-mitochondrial oxygen consumption.

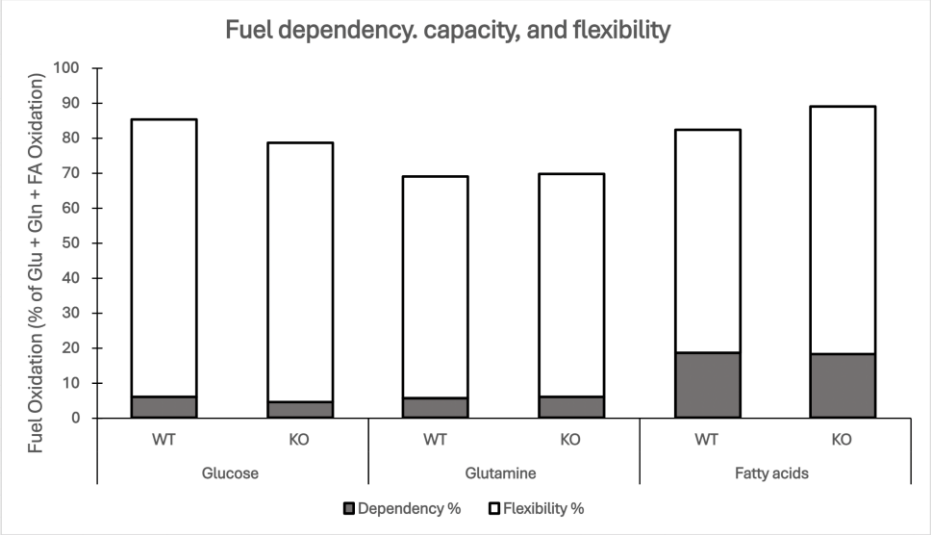

Figure 4. Fuel dependency, capacity, and flexibility for glucose, glutamine, and fatty acid oxidation pathways in wildtype (n=1) and knockout (n=1) MEF cell lines determined with data gathered using the Seahorse XF Mito Fuel Flex Test. The Fuel Flex Test can be used to determine the presence of fuel type preference between glucose, glutamine, and fatty acids. Dependency is defined as the quantitative measurement of cells' reliance on the pathway of interest to maintain basal respiration. It is measured by first inhibiting the pathway of interest followed by the two alternative pathways. Capacity reflects the level of oxidation of the fuel of interest when alternate fuel pathways are inhibited. It is measured by inhibiting the two alternative pathways followed by the pathway of interest. Flexibility is calculated as the magnitude of difference between the fuel Dependency and the fuel Capacity. It indicates whether the cells' mitochondria can increase the activity of alternative fuel pathways to compensate for inhibition of the pathway of interest. The % values for each quantity were averaged from six technical replicates per quantity per cell line, with unphysical values and outliers (outside the mean±2SD) removed. Dependency % and Capacity % were calculated using equations from the user manual.

Previous work in the group provided evidence that CALHM2 levels increased alongside cardiomyocyte metabolic maturation, suggesting a putative role in the shift from primarily glycolysis to primarily FAO in the developing murine heart. To test this, we performed two Mito Stress Tests (one of which represented in Figure 3) and two Fuel Flex Tests (one of which is represented in Figure 4). Contradicting what was expected from prior work, there was no significant difference in oxidative capability found between the wildtype and *Calhm2*-knockout MEF cell lines (Figure 3), nor was there a significant difference found in FAO dependency or capacity (Figure 4).

One possible explanation for the surprising result could be that the inhibitors used (UK5099, BPTES, Etomoxir for the Fuel Flex Test) had lost efficacy due to having been frozen for some time after reconstitution. In every well in this assay being studied for fuel Dependency, it was found that only very small changes in OCR occurred upon injection 1 (wherein significant changes should be expected upon inhibition of a fuel pathway); the small drops in OCR could reflect merely changes in response to injection of media rather than pathway inhibition.

Departures from original project plan

Three KOMP PCR genotyping experiments were performed, with one partially successful attempt confirming the expected genotype of two cell lines.

Several Western blots failed to detect any CALHM2 protein due to an unreliable antibody despite 1) using Tris-buffered saline instead of PBS-T, 2) using 1% BSA instead of 5% skim milk powder, and 3) an optimisation protocol experimenting with different buffers, whether reducing agent was present or not, and whether the sample was boiled or not; these affected the specificity of antibody binding but did not show any binding to monomeric CALHM2 (32kDa). While genotyping determines the presence of the gene and qRT-PCR determines the presence of RNA—of which neither equate to protein quantity—Western blotting quantifies protein expression. Thus, a successful immunoblot demonstrating the presence or absence of CALHM2 in the MEF cell lines would have been a more powerful confirmation compared to gene- or expression-directed assays (ie. genotyping or qRT-PCR) because the overall goal of the project was to investigate the impact of CALHM2 protein on FAO capacity.

Future directions for the project

As quantification of gene expression via qRT-PCR does not equate to the presence of protein product, achieving reliably successful Western blots (through discovery of a high-quality antibody and/or further attempts at optimisation) should be a priority of future work. An alternative to this could be to pursue the use and optimisation of methods such as RNAseq. Regarding the Seahorse assays, one clear direction to take is to reattempt the experiments with freshly reconstituted inhibitors to ensure that changes in OCR reflect real inhibition of the pathways of interest. Another direction for consideration would be to continue to study the suitability of MEFs as the model cell system for the group.

Acknowledgements

I am very grateful to Dr Thomas Nicol for overseeing my work daily, teaching me new lab skills, and patiently answering my questions about our work as well as about life in academia. I am very grateful to Professor Nicola Smart for giving me the opportunity to work with her group. I am also grateful to the Heather Group, who were friendly and easy to work with (and generous with their coffee). Finally, I am especially grateful to the Biochemical Society for funding this project (thereby alleviating much of my financial stress) and requiring the authorship of this end-of-project report (thereby ensuring that I gained experience not only with wet lab research, but also with writing about it in a coherent manner).

During my studentship with the Smart Group, I have learned techniques in cell culture, qRT-PCR, and Agilent Seahorse extracellular flux analysis, the last of which I am especially grateful for. Additionally, I have gained confidence in DNA, RNA, and protein extraction as well as Western blotting. I have gained innumerable skills in experimental design, optimisation, and data analysis

within the context of an academic lab, which has provided ample opportunity for me to think and learn independently while recognising the importance of collaboration between scientists. Notably, I have also tested and improved my resilience in the face of unexpected obstacles, requiring adaptability and dedication to the project goals. Completing this studentship has taught me far more about biochemical techniques and academic life than I had anticipated. It has given me much food for thought as to my personal career development and impressed upon me both the excitement and difficulty of an academic career, which I will no doubt find valuable as I pursue a new research project after my final year of undergraduate studies.