

SIRT4 is a regulator of human skeletal muscle fatty acid metabolism influencing inner and outer mitochondrial membrane-mediated fusion

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ABSTRACT

Objective: The mitochondrial phenotype, governed by the balance of fusion-fission, is a key determinant of energy metabolism. The inner and outer mitochondrial membrane (IMM) fusion proteins optic atrophy 1 (OPA1) and Mitofusin 1 and 2 (Mfn1/2) play an important role in this process. Recent evidence also shows that Sirtuin 4 (SIRT4), located within the mitochondria, is involved in the regulation of fatty acid oxidation. The purpose of this study was to determine if SIRT4 expression regulates inner and outer mitochondrial-mediated fusion and substrate utilization within differentiated human skeletal muscle cells (HskMC).

Material and methods: SIRT4 expression was knocked down using small interfering RNA (siRNA) transfection in differentiated HskMC. Following knockdown, mitochondrial respiration was determined by high-resolution respirometry (HRR) using the Oroboros Oxygraph O2k. Live cell confocal microscopy, quantified using the Mitochondrial Network Analysis (MiNA) toolset, was used to examine mitochondrial morphological change. This was further examined through the measurement of key metabolic and mitochondrial morphological regulators (mRNA and protein) induced by knockdown.

Results: SIRT4 knockdown resulted in a significant decrease in LEAK respiration, potentially explained by a decrease in ANT1 protein expression. Knockdown further increased oxidative phosphorylation and protein expression of key regulators of fatty acid metabolism. Quantitative analysis of live confocal imaging of fluorescently labelled mitochondria following SIRT4 knockdown supported the role SIRT4 plays in the regulation of mitochondrial morphology, as emphasized by an increase in mitochondrial network branches and junctions. Measurement of key regulators of mitochondrial dynamics illustrated a significant increase in mitochondrial fusion proteins Mfn1, OPA1 respectively, indicative of an increase in mitochondrial size.

Conclusions: This study provides evidence of a direct relationship between the mitochondrial phenotype and substrate oxidation in HskMC. We identify SIRT4 as a key protagonist of energy metabolism via its regulation of IMM and OMM fusion proteins, OPA1 and Mfn1. SIRT4 knockdown increases mitochondrial capacity to oxidize fatty acids, decreasing LEAK respiration and further increasing mitochondrial elongation via its regulation of mitochondrial fusion.

1. Introduction

Sirtuin 4 (SIRT4) is an NAD⁺-dependent deacetylase and ADP-ribosyltransferase located in the mitochondrial matrix that is known to have a wide range of effects on metabolic pathways, the tricarboxylic

acid (TCA) cycle, glycolysis, reactive oxygen species (ROS), oxidative phosphorylation and more directly, fatty acid metabolism [1]. Despite this, the role and function of SIRT4 within humans is poorly understood at present.

Human skeletal muscle fatty acid metabolism is dependent on the

Abbreviations: Inner mitochondrial membrane, IMM; Outer mitochondrial membrane, OMM; Optic atrophy 1, OPA1; Sirtuin 4, SIRT4; Mitofusin 1, Mfn1; human skeletal muscle cells, HskMC; High-resolution respirometry, HRR.

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functional capacity of the mitochondria [2], in particular the balance between mitochondrial biogenesis, mitochondrial dynamics, and degradation/removal (mitophagy). Mitochondrial dynamics plays a crucial role in determining the morphology, phenotype, and integrity of mitochondria [3–7]. This process is regulated by both inner (IMM) and outer mitochondrial membrane (OMM) proteins involved in the division (fission) and binding of mitochondria (fusion) [7,8].

Mitochondrial fission is initiated by the translocation of dynamin-related protein 1 (Drp1) from the cytosol to the OMM where it binds to anchor proteins such as mitochondrial fission protein 1 (Fis1), mitochondrial fission factor-1 (Mff-1), mitochondrial dynamics protein 49 (Mif49) and/or mitochondrial dynamics protein 51 (Mif51), forming ring-like structures at future sites of division [9–11]. Abscission is subsequently culminated via the action of dynamin 2 (DNM2) and the endoplasmic reticulum (ER-mitochondria contact sites – ERMCS) dividing the mitochondrial reticula into multiple, fragmented mitochondria [9,12–18].

Mitochondrial fusion is a two-step process regulated by IMM and OMM proteins such as Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2) and Optic Atrophy 1 (OPA1). An upregulation of mitochondrial fusion increases mitochondrial oxidative capacity and is pivotal in the management of cellular stress through cross-complementation. An increase in fusion allows for greater exchange of proteins and lipids, thereby aiding mitochondria in their adaptation to adverse environmental stressors [7,19]. Dysfunction in mitochondrial fusion has been proposed to impair skeletal muscle lipid oxidation, increasing cellular lipid accumulation potentially leading to metabolic disease progression [20].

OPA1 is a specialised dynamin-like GTPase on the inner mitochondrial membrane (IMM) regulated by the metalloendopeptidase, OMA1, and the i-AAA protease, YME1L, which cleaves the IMM bound long-OPA1 (L-OPA1) to the soluble short-OPA1 (s-OPA1) [4,21–23]. OPA1 mediated mitochondrial fusion occurs once Mfn1/2 tether and fuse to the OMM of an adjacent mitochondrion. L-OPA1, which is tethered to the inner membrane space (IMS), binds to and fuses with neighboring L-OPA1 on adjacent mitochondria [24]. This fusion process helps give the cristae its curvature, enhancing oxidative phosphorylation (OXPHOS) by allowing for a greater distribution of OXPHOS complexes [25], decreasing the diffusion of substrates and electron transfer [24,26] and further improving mitochondria-to-mitochondria communication. Loss of OPA1 results in a widening of the cristae lumen and junction, a reduction in IMM potential, an increase in basal mitochondrial calcium level and an induction of apoptosis [15,17,27,28].

Given their pivotal role in energy metabolism, sirtuins may also play a role in regulating mitochondrial morphology [29–32]. SIRT3 has already been shown to deacetylate OPA1 during times of stress in an attempt maintain the mitochondrial structure [33]. With the benefits of mitochondrial elongation on cellular metabolism, particularly to fat metabolism, the impact of SIRT4 on mitochondrial function in human skeletal muscle requires further investigation. The purpose of this study was to investigate the role of SIRT4 on the regulation of outer and inner mitochondrial membrane mediated fusion and whether deficiency in SIRT4 may enhance the mitochondrial metabolism of fatty acids *in vitro*.

2. Material and methods

2.1. Human skeletal muscle culturing

Primary human skeletal muscle cells (HskMC) (ATCC #PCS-950-010) were cultured at 37 °C, 5% CO₂ and maintained in the mid-exponential phase of their growth cycle in mesenchymal stem cell basal media (ATCC #PCS-500-030™) supplemented with the primary skeletal muscle growth kit (ATCC #PCS-950-040™) consisting of L-glutamine (10 mM), recombinant (rh) Embryonic Fibroblast Factor (EGF) (5 ng/ml), dexamethasone (10 μM), rh Fibroblast Growth Factor-basic (FGF-b) (5 ng/ml), rh insulin (25 μg/ml) and stem cell qualified

fetal bovine serum (FBS) (4%). The cells were maintained in the mid-exponential phase of their growth cycle for the entirety of the study, with all samples derived from the cells representative of this phase of the cell cycle.

Once HskMC reached 70–80% confluence, cells were detached and passaged using sterile trypsin solution (0.25% trypsin (Gibco™, 15090046), 0.01% EDTA (Sigma-Aldrich, E9884) in sterile phosphate buffer saline (PBS)) and subsequently neutralized using trypsin neutralizing solution (ATCC #PCS-999-004). Cell viability was subsequently measured using the trypan blue technique prior to seeding at 0.5×10^5 per well of a 6-well plate. The same cell seeding density was used for each experiment.

Once the HskMC reached 80–90% confluence, differentiation was induced by removing the existing culture media, washing cells with PBS to remove any residual FBS containing media, and replacing it with differentiation media (ATCC, #PCS-950-050). The cells were cultured at 37 °C and 5% CO₂, with media exchanged on days 3, 6, and 9. Based on characterization studies (measurement of myotube elongation and gene expression of targets of immaturity/maturity – see Supplementary Data, Fig. 2) cells were deemed maturely differentiated myotubes after day 10–12 and fit for experimental analyses.

2.2. SIRT4 siRNA

All experimentation was conducted using fully differentiated myotubes with RNA interference (RNAi) using small interfering RNA (siRNA) transfection conducted on primary HskMC differentiated between 10 and 12 days.

To investigate the influence of SIRT4, differentiated primary HskMC were transfected using SIRT4 Silencer Select Pre-Designed siRNA (Thermo Fisher Scientific Inc., s23764 and s23765) and Lipofectamine 3000 transfection reagent (Invitrogen™, L3000001) as per the manufacturer's instructions. All siRNA reagents were made to 100 μM using sterile nuclease free water in sterile conditions. From this 100 μM stock solution all siRNA silencer selects were made to 10 μM using sterile nuclease free water in sterile conditions. These 10 μM solutions were used for all siRNA experiments.

Briefly, cells which had been differentiated for 10–12 days were cultured with OptiMEM I Reduced Serum Medium (Gibco™, 31,985,047) containing 75 nM of a duplex formulation (s23764 and s23765) and Lipofectamine 3000 for 24 h. Optimal transfection reagent, siRNA concentration and incubation time were assessed to significantly knock down SIRT4 transcriptionally and translationally prior to any analysis. Negative control siRNA (NC-siRNA) groups utilizing a Silencer Select Pre-Designed siRNA scramble (Thermo Fisher Scientific Inc., 4,390,843) at 75 nM in place of the duplex were run in parallel in all experiments. Following incubation, the cells transcriptional and translation expression, respiratory capacity, and mitochondrial phenotype were assessed through different downstream assays.

2.3. Protein expression – Western blot

Protein was extracted and quantified using the Bradford technique before being prepared at 0.5 mg/ml within 5× Laemmli buffer and homogenisation buffer (Okadiaz buffer (200uM), 50× protease inhibitor (Sigma Aldrich, P8340), DTT (100 mM), sodium orthovanadate (100 mM) and PMSF (100 mM)). Sample proteomes were subsequently separated on 4–12% precast gradient gels and transferred to polyvinylidene difluoride (PVDF) membranes (iBlot™ TM Device, Thermo Fisher Scientific, MA, USA). Membranes were blocked in 5% milk (Bio-Rad, 170–6404) in TBS (1×) for 2 h before incubation in primary antibody of target proteins, which were prepared in the Odyssey® blocking buffer and antibody dilution buffer (99.8% Tris-Buffered Saline, 0.1% sodium azide, 0.1% bovine serum albumin), overnight at 4 °C. Following primary incubation overnight, the membrane was washed six times for 10 min in TBS-tween-20 (0.1%) detergent prior to incubation with goat

anti-rabbit/mouse IgG (H + L)-Horseradish peroxidase (HRP) conjugate secondary (Bio-Rad, Cat:170–6515 & 170–6516) for 2 h protected from light. Membranes were subsequently washed with TBS-tween-20 (0.1%) and protein expression detected using enhanced chemiluminescence (ECL) (Bio Rad, Clarity Western ECL Substrate, #1705061) and quantified by densitometry using the TotalLab Quant™ software (v1.0). Protein loading was normalised to α -Tubulin, while a biological (internal) control sample was used to control gel-to-gel variation. Positive controls for all targets measured were generated to ensure the appropriate western blot reference band was captured. A list of the antibodies employed, their sources, working concentrations, and positive controls can be found in Appendices, Supplementary Data, Table 1.

2.4. Gene expression – RT-qPCR

RNA was isolated from myotubes using the Tri reagent guanidinium thiocyanate-phenol-chloroform (Sigma-Aldrich, T9424) extraction method [34]. RNA was subsequently quantified using a Nanodrop®ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and reverse transcribed into cDNA in equal amounts of High-Capacity cDNA Reverse Transcription Kit 200 Rxn (Thermo Fisher Scientific Inc., 4,368,814) within a PCR thermocycler (Bio-Rad™, USA) as per the manufacturer's instructions. All cDNA samples were subsequently quantified using the NanoDrop® ND-1000 system and made to 200 ng/ μ l using nuclease-free water.

RT-qPCR was subsequently performed on all extracted cDNA samples. All RT-qPCR reactions were conducted on cDNA samples prepared within a mastermix consisting of Syber Green (FastStart Essential DNA Green Master, Roche™, 06402712001), nuclease-free water and the forward (10 μ M) and reverse (10 μ M) primer of the target of interest as per the manufacturer's instructions. The LightCycler® 96 instrument (Roche™) was used for all RT-qPCR reactions with quantification cycle (Cq) values analysed using the standard delta-delta Cq method ($\Delta\Delta$ Cq). GAPDH was used as a loading control to normalize all probed targets.

All primers were custom designed in-house and synthesized at 0.025 μ M in dry format by Merck™. Primers were subsequently made to 100 μ M using nuclease-free water in a RNase free environment. Quality control was performed on all primers to ensure consistency between experiments was managed by maintaining Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline standards with primer efficiency accepted between 90 and 110%. All samples were measured within RNase free conditions. A list of primers used within this study and their sequences can be found in Appendices, Supplementary, Table 2.

2.5. Mitochondrial and nuclear staining – Immunofluorescence

2.5.1. Mitochondrial staining

MitoTracker Green FM (Invitrogen™, M7514) was used for the quantification of mitochondrial content. MitoTracker Green FM was prepared and stored at 1 mM using sterile dimethyl sulfoxide (DMSO) as per the manufacturer's instructions. Briefly, 150 nM MitoTracker Green FM was prepared in differentiation medium (ATCC #PCS-950-050™) as per manufacturer's instructions. HSkMC; grown and differentiated for 10 days on 35 mm imaging dishes with a polymer coverslip bottom (Ibidi GmbH™, μ -Dish 35 mm, 81,156), were prepared for this assay by removing media and washing the cells twice in phosphate buffer saline (PBS), before incubating in darkness in fresh differentiation media supplemented with 150 nM Mitotracker Green FM for 30 min at 37 °C, 5% CO₂. Following incubation, media was removed, cells were washed with PBS twice, before counter staining using NucBlue Live Stain (Thermo Fisher Scientific Inc., Hoechst 33342 dye, R37605) for nuclei content. Cells were refed with human skeletal muscle differentiation medium prior to confocal microscopy imaging.

2.5.2. Confocal microscopy

Live cell imaging was performed on mitochondrial stained cells using a Leica DM IRE2 confocal microscope (63 $\times$ with 4 $\times$ Zoom), with environmental control (37 °C with humidified 5% CO₂). Random fields within each well were selected to measure mitochondrial tubulation per cell. Photomultiplier Tube 1 (PMT1) was set between 405 nm and 488 nm for nuclei imaging, while PMT2 was set between 500 nm and 550 nm for mitochondrial imaging. Further subsequent image processing and deconvolution was performed using AutoDeBlur.

2.5.3. Mitochondrial network analysis (MiNA)

Using the Mitochondrial Network Analysis (MiNA) toolset in ImageJ, a macro tool for analyzing mitochondrial network morphology in mammalian cell culture [35], the mitochondrial networks were analysed as per Valente et al. 2017 [35]. This toolset recognizes two distinct object types; individuals (structures with no junctions) and networks (structures with at least one junction). Networks are broken into; branch, junctions, slabs, and end-points. See Valente et al. 2017, for graphical representation of the image preparation process within ImageJ and terminology used.

2.6. Mitochondrial respiration – Oroboros Oxygraph O2k

Differentiated HSkMC respiration was measured in permeabilized HSkMC at 37 °C in each chamber of an Oroboros Oxygraph-O2k containing 2 ml MiR05 (EGTA 0.5 mM, MgCl₂·6H₂O 3 mM, taurine 20 mM, KH₂PO₄ 10 mM, HEPES 20 mM, BSA 1 g liter⁻¹, potassium-lactobionate 60 mM, mannitol 110 mM, dithiothreitol 0.3 mM, pH 7.1, adjusted with 5 M KOH). Oxygen concentration within each chamber was set at 200 nmol O₂/ml. The software DatLab 5 (Oroboros Instruments GmbH, Innsbruck, Austria) was used for data acquisition at 2 s time intervals. Following siRNA SIRT4 knockdown of human skeletal muscle myotubes, cells were prepared for their Oroboros-O2k SUIT protocol.

In sterile conditions differentiation media was removed from HSkMCs differentiated for 10 days. Cells were subsequently trypsinized and further neutralized using skeletal muscle differentiation media (ATCC #PCS-950-050™), centrifuged at 1000 rpm for 5 min and resuspended in 5 ml mitochondrial respiration medium, MiR05 [36]. Following cell count, 2.5 ml of counted cell suspension was pipette into the two empty Oroboros-O2k chambers, previously calibrated with MiR05, for experimentation. All respiratory data was normalised to O₂ flow per cell [pmol/(s*ml)].

The fatty acid (FA) substrate-uncoupler-inhibitor-titration (SUIT) protocol for high-resolution respirometry was performed in digitonin (10 μ g·10⁻⁶ cells/chamber)-permeabilised skeletal muscle myotubes. Permeabilization only occurred in cells for Oroboros Oxygraph-O2k experimentation. LEAK (state 4) respiration was measured by the addition of palmitoyl-carnitine (0.04 nM/chamber) and malate (5 mM/chamber) (PalM). Oxidative phosphorylation (state 3) (OXPHOS) was then measured with the addition of adenosine diphosphate (ADP) (2.5 mM/chamber). The experiment proceeded with the addition of malonic acid (Mna) (5 mM/chamber) and Antimycin A (Ama) (0.0025 mM/chamber) to inhibit complex II and III for the calculation of residual oxygen consumption (ROX). All mitochondrial respiratory states were corrected for ROX. Data was subsequently represented as O₂ flow per cell, as per [37], relative to control [pmol/(s*ml)].

2.7. Statistical analysis

Data is presented as a mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) with pair-wise comparisons was used to determine the differences between treatments for cell culture experiments. Where a main effect was found, the student Bonferroni post-hoc test was used to determine where the difference was. Experiments were carried out with a minimum of three replicates in each experimental group for analysis. All siRNAs were designed to directly repress the

transcription and translation of the SIRT4 gene. It was confirmed that the sequence of the negative control siRNA (NC-siRNA) did not directly interfere with the expression of SIRT4 only (Fig. 1). As such, data of SIRT4 transcriptional and translational expression is presented normalised to both the non-transfected control (NT-Control) and the NC-siRNA separately. Similarly, mitochondrial functional data are expressed relative to both NT-Control and NC-siRNA (Fig. 2), whilst MiNA data has not been normalised (Fig. 3). The expression of all other transcriptional and translational are compared against the NT-Control (Fig. 4), given the indirect effect the scramble may have on other non-SIRT4 markers. All statistical analysis was performed using GraphPad Prism with significance set at $p < 0.05$.

3. Results

3.1. Transcriptional and translational expression of SIRT4 with SIRT4 knockdown

Incubation of differentiated myotubes in 75 nM duplex SIRT4 siRNA (s23764 and s23765) for 24 h with Lipofectamine 3000 transfection reagent (i.e. Duplex) resulted in a significant decrease in mRNA and protein SIRT4 expression when expressed relative to the NT-Control ($p < 0.005$) and NC-siRNA ($p < 0.05$).

3.2. SIRT4 regulates skeletal muscle oxidative phosphorylation via its direct regulation of LEAK respiration

Differentiated HSkMC mitochondrial respiration data, expressed as O_2 flow per cell relative to the NT-Control and the NC-siRNA [pmol/(s*mil)], is presented in Fig. 2 (A-D). SIRT4 knockdown (Duplex) resulted in a significant decrease in LEAK (state 4) respiration when compared to the NT-Control ($p < 0.05$) (Fig. 2. A) and the NC-siRNA ($p < 0.005$) (Fig. 2. C). We noted a significant increase in SIRT4 knockdown (Duplex) ADP-stimulated oxidative phosphorylation (state 3) when compared to the NT-Control ($p < 0.05$) (Fig. 2. B) and the NC-siRNA ($p < 0.05$) (Fig. 2. D). Protein expression of adenosine nucleotide translocase 1 (ANT1) was significantly decreased in the SIRT4 knockdown (Duplex) samples in comparison to the NT-Control ($p < 0.0005$) (Fig. 2. E). Further, phosphorylation of Acetyl-CoA Carboxylase at serine 79 (pACC^{Ser79}); which is used as a surrogate for AMPK activity, was increased following SIRT4 knockdown (Duplex) ($p < 0.05$) in comparison to the NT-Control (Fig. 2. F). These results suggest a direct role of SIRT4 on ANT1 and LEAK respiration as a potential regulator of ADP:ATP ratio and subsequently fatty acid metabolism. The phosphorylation of ACC at serine 79 acts to change in state of ACC, inactivating it, lowering the levels of the Malonyl-CoA. This subsequent decrease in Malonyl-CoA may be acting to alleviate Malonyl-CoA's allosteric

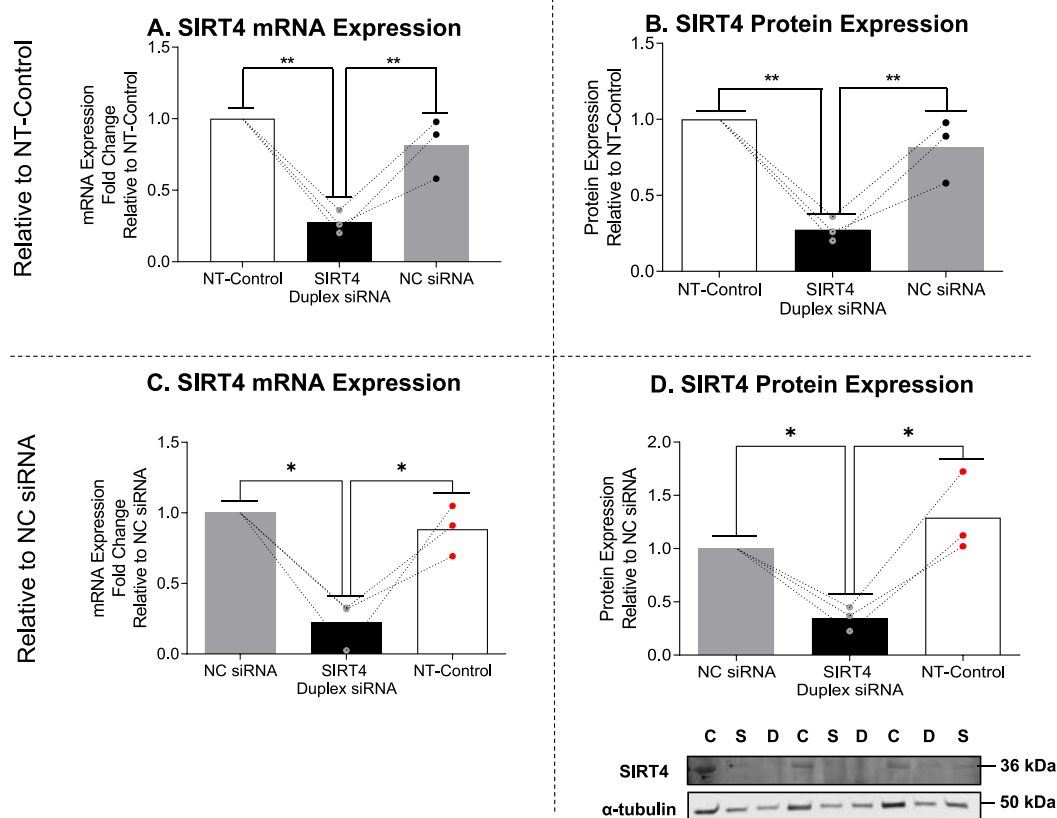


Fig. 1. Transcriptional and translational expression of SIRT4 following SIRT4 knockdown. Total SIRT4 mRNA expression (A) in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) siRNA HSkMC myotubes quantified by RT-qPCR ($n = 3$) relative to NT-Control. Total SIRT4 protein expression (B) in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes quantified by western blot ($n = 3$) relative to NT-Control. Total SIRT4 mRNA expression (C) in NC-siRNA (75 nM), SIRT4 Duplex (75 nM) siRNA and NT-Control HSkMC myotubes quantified by RT-qPCR ($n = 3$) relative to NC-siRNA. Total SIRT4 protein expression (D) in NC-siRNA, SIRT4 Duplex (75 nM) siRNA and NT-Control HSkMC myotubes quantified by western blot ($n = 3$) relative to NC-siRNA. White bars, NT-Control; black bars, SIRT4 Duplex (75 nM) siRNA; grey bars, NC-siRNA (75 nM). Individual response represented by dots; SIRT4 Duplex (75 nM) siRNA (grey), NC-siRNA (75 nM) (black), NT-Control (red). Gene expression was normalised against GAPDH mRNA expression of same sample. Protein expression was normalised against α -Tubulin expression of all samples. Blots representative of all samples. Data are expressed as mean $\pm$ SD. ** significant difference between groups, $p < 0.005$, *significant difference between groups, $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

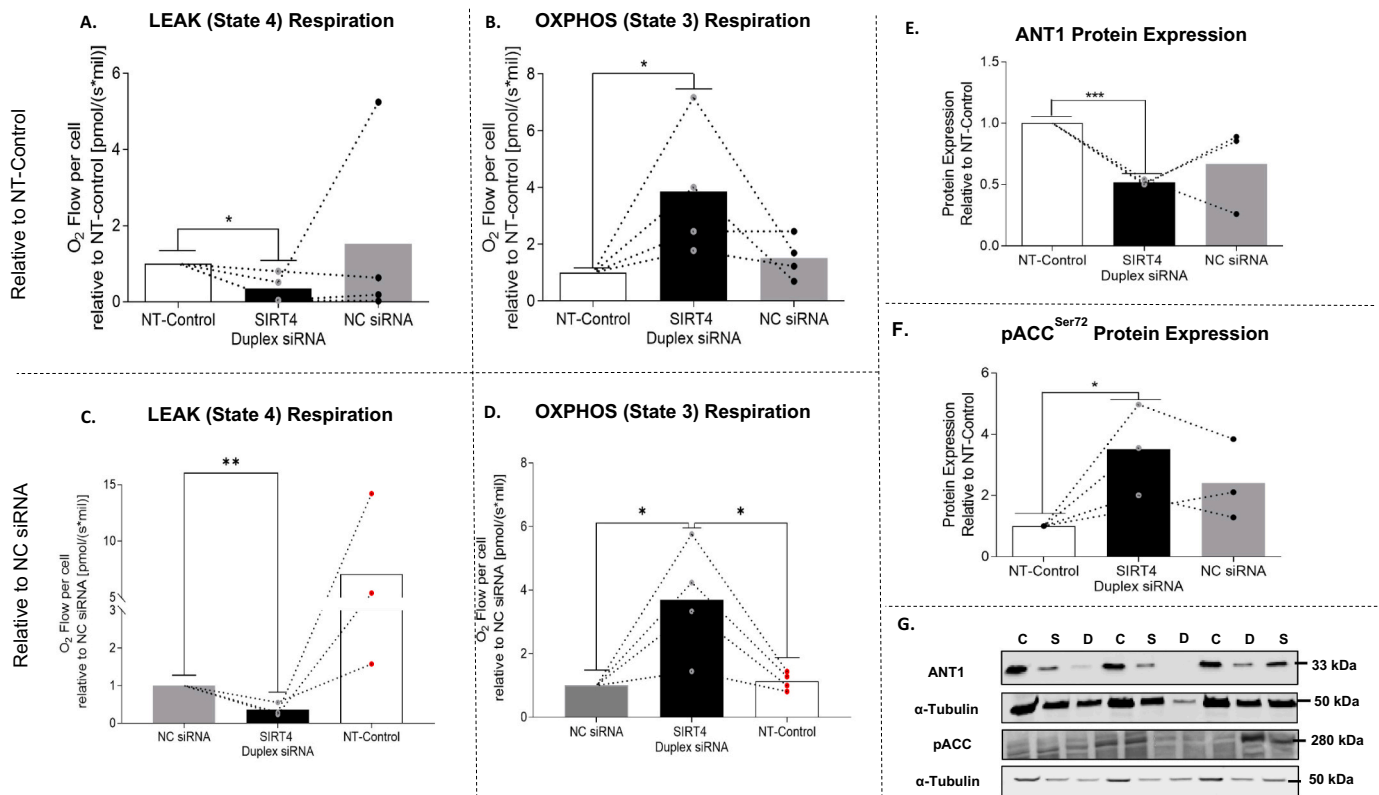


Fig. 2. SIRT4 regulates skeletal muscle oxidative phosphorylation via its direct regulation of LEAK respiration. LEAK (A) and OXPHOS (B) respiration in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes quantified by HRR ($n = 4$) relative to NT-Control. LEAK (C) and OXPHOS (D) respiration in NC-siRNA (75 nM), SIRT4 Duplex (75 nM) siRNA and NT-Control HSkMC myotubes quantified by HRR ($n = 4$) relative to NC-siRNA. Oxygen flow was normalised to cell number of the same sample. (E) Total ANT1 and (F) pACC^{Ser72} protein expression in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes relative to NT-Control quantified by western blot ($n = 3$). Protein expression was normalised against α -Tubulin expression of same sample. Gene expression was normalised against GAPDH mRNA expression of same sample. (G) Blots of all targets with respective loading controls and molecular weight in kilodaltons (kDa). White bars, NT-Control; black bars, SIRT4 Duplex (75 nM) siRNA; grey bars, NC-siRNA (75 nM). Individual response represented by dots; SIRT4 Duplex (75 nM) siRNA (grey), NC-siRNA (75 nM) (black), NT-Control (red). Data are expressed as mean $\pm$ SD, * significant difference between groups, $p < 0.05$, *** significant difference between groups, $p < 0.0005$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibition of CPT1b, leading to increased CPT1b expression (see Supplementary Data, Fig. 9 & 10), subsequently increasing lipid oxidation. Without SIRT4, the regulation of ANT1 is absent leading to an increase in pACC^{Ser79} mediated OXPHOS.

3.3. SIRT4 directly regulates mitochondrial morphology increasing mitochondrial network branches and junctions

Assessment of mitochondrial morphology following SIRT4 knockdown using confocal microscopy can be seen in Fig. 3. A. Further analysis of mitochondrial networks using the Mitochondrial Network Analysis (MiNA) toolset in ImageJ indicated a significant increase in mitochondrial network branches (Fig. 3. B) following SIRT4 knockdown (Duplex) when compared against the NT-Control ($p < 0.05$) and the NC-siRNA ($p < 0.05$). Further we noted a significant increase in mitochondrial network junctions (Fig. 4. C) following SIRT4 knockdown (Duplex) when compared against the NT-Control ($p < 0.005$) and the NT-Control ($p < 0.05$). This data is further complimented within Fig. 3. D through H suggestive that a decrease in SIRT4 results in a more elongated mitochondrial network.

3.4. SIRT4 knockdown impacts the regulation of other mitochondrial fusion and fission targets

A series of mitochondrial fusion targets were measured for both mRNA and protein expression using RT-qPCR and western blot, the

profiles of which are present in Fig. 4. We observed a significant increase in the protein and mRNA expression of Mfn1 (Fig. 4. A & B) with SIRT4 knockdown (Duplex) in comparison to the NT-Control ($p < 0.05$). These increases in regulators of outer mitochondrial membrane fusion were followed suit by an increase in the mRNA and protein expression of inner mitochondrial membrane fusion target, OPA1 ($p < 0.05$) (Fig. 4. D & E), along with its long isoform L-OPA1 ($p < 0.05$) (Fig. 4. I). There was no significant change in Mfn2 protein (Fig. 4. F) or mRNA expression (Fig. 4. G) with SIRT4 knockdown (Duplex). Additionally, there was no significant change in OMA1 (Fig. 4. C) or YME1L (Fig. 4. H) mRNA expression, known regulators of OPA1 expression, following SIRT4 knockdown (Duplex).

Mitochondrial fission targets were also measured for mRNA and protein expression following SIRT4 knockdown (Duplex). We observed no significant change in Drp1 protein (Fig. 4. K) or mRNA (Fig. 4. N) expression with SIRT4 knockdown (Duplex). Mff mRNA expression (Fig. 4. O) was significantly increased in comparison to both the NT-Control ($p < 0.05$) and NC-siRNA ($p < 0.05$). The mRNA expression of MiD49 (Fig. 4. M) was also significantly increased in the SIRT4 knockdown (Duplex) samples comparison to the NT-Control ($p < 0.05$). We noted no change in mRNA expression of either Fis1 (Fig. 4. L) or MiD51 (Fig. 4. P) with SIRT4 knockdown (Duplex).

We are unable to decipher if SIRT4's regulation of mitochondrial fusion is a primary or secondary result of SIRT4's governance of oxidative phosphorylation. However, given a more tubulated mitochondrial phenotype has been shown to lead to a prioritisation in fatty acid

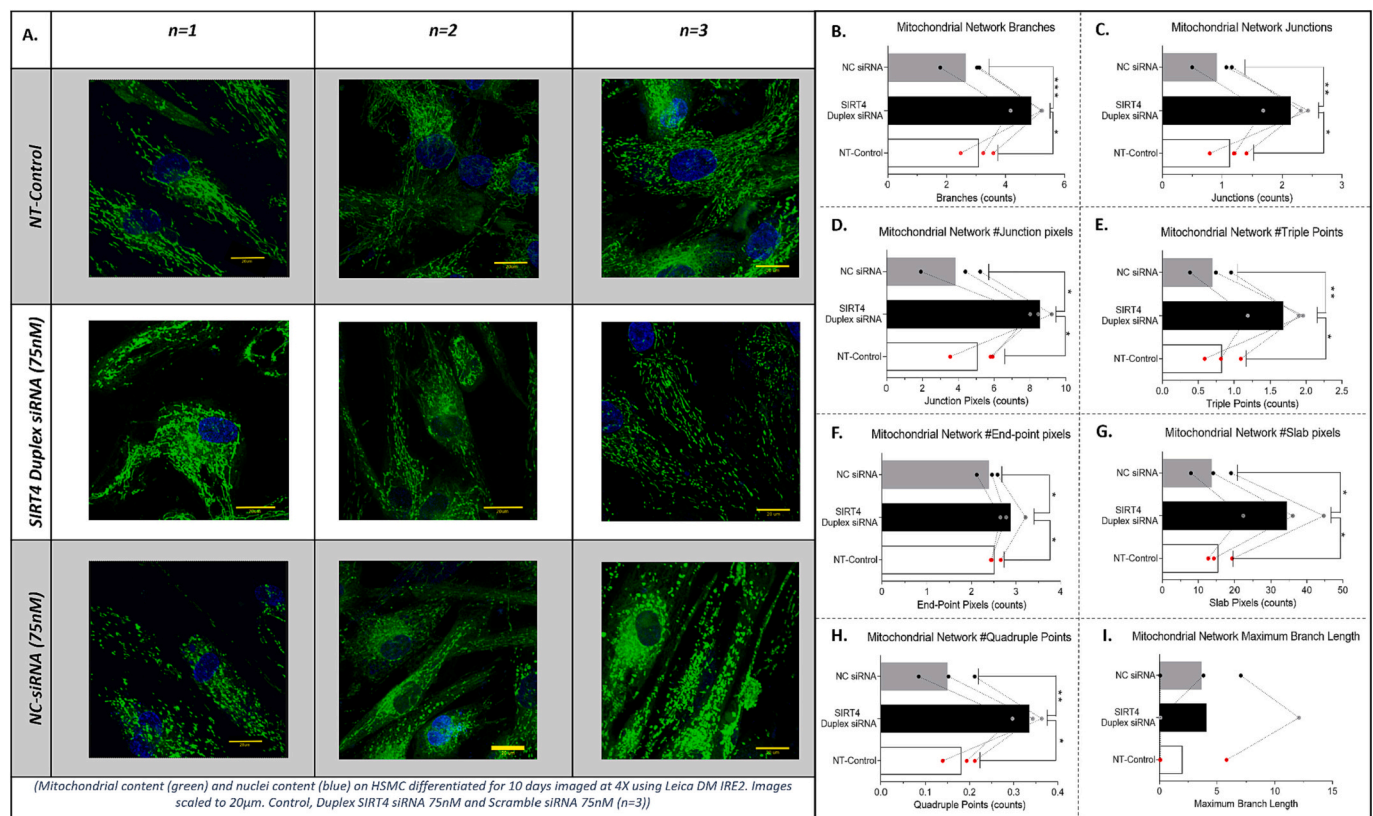


Fig. 3. SIRT4 regulates mitochondrial morphology through its direct regulation of mitochondrial fusion. (A) Mitochondrial content (green) and nuclei content (blue) in human skeletal muscle myotubes differentiated for 10 days and imaged at 4× using Leica DM IRE2. Images scaled to 20 µm. NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA HSKMC myotubes ($n = 3$). Mitochondrial Network Analysis (MiNA) using ImageJ. Mitochondrial network branches (B), Mitochondrial network junctions (C), Mitochondrial network #junction pixels, (E) Mitochondrial network #triple points, (F) Mitochondrial network #end-point pixels, (G) Mitochondrial network #Slab pixels, (H) Mitochondrial network #quadruple points and (I) Mitochondrial network maximum branch length are presented in counts. White bars, NT-Control; black bars, SIRT4 Duplex (75 nM) siRNA; grey bars, NC-siRNA (75 nM). Individual response represented by dots; SIRT4 Duplex (75 nM) siRNA (grey), NC-siRNA (75 nM) (black), NT-Control (red). Data are expressed as mean $\pm$ SD, * significant difference between groups, $p < 0.05$, ** significant difference between groups, $p < 0.005$, *** significant difference between groups, $p < 0.0005$ ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

oxidation [38–42], as our data suggests, that SIRT4 may be integral in regulating the switch in substrate use during cellular metabolism.

4. Discussion

Sirtuin 4 (SIRT4) knockdown resulted in functional, structural, transcriptional and translational adaptations to the mitochondria. Such changes epitomize the importance of SIRT4 in the regulation of cell metabolism within HSKMC. In particular, we identified the nuclear encoded, SIRT4, to be integral in the transcriptional regulation of mitochondrial morphology via its control of inner and outer mitochondrial membrane fusion proteins, OPA1 and Mfn1 (also nuclear encoded). We demonstrate how SIRT4 knockdown acts to increase the translational and transcriptional expression of these markers and further increases mitochondrial network junctions and branches indicative of increases in mitochondrial elongation. This increase in mitochondrial size is further emphasized by the observed decrease in LEAK respiration and improvement in fatty acid oxidation.

This study is the first indication of SIRT4's inhibitory regulation of mitochondrial fusion, emphasizing SIRT4's governance of the mitochondrial phenotype. Previous data has indicated a specific role SIRT4 has in relation to OPA1. Work by Lang et al. [43] suggested that moderate overexpression of SIRT4 increased OPA1 expression, further promoting mitochondrial fusion, in human embryonic kidney 293 (HEK293) cells. However, our data illustrates that these effects may be tissue specific, exemplified by how knockdown of SIRT4 in HSKMC

increased the transcriptional and translational expression of OPA1, along with its long isoform (L-OPA1). This was further supported by the increase in the transcriptional and translational expression of outer mitochondrial membrane fusion target, Mfn1. Additionally, we observed an increase in the transcriptional expression of Mff and MiD49 (also known as Mitochondrial Elongation Factor 2, MIEF2), both of which have been referenced to be involved in both fission but also fusion processes by regulating the activity and expression of mitochondrial fission marker Drp1 [44], which did not change following knockdown. This pro-fusion phenotype was further supported by our microscopy data which pointed to an increase in mitochondrial network branches and junctions with SIRT4 knockdown. Qualitatively, we observed that knockdown of SIRT4 resulted in an increase in more elongated mitochondria in comparison to both the NC-siRNA and NT-Control. This is further supported by our quantitative analysis of mitochondrial networks using the Mitochondrial Network Analysis (MiNA) toolset [35], which recognised an increase in mitochondrial network branches and junctions, indicative of an increase in mitochondrial elongation following knockdown [35]. These results further support the inhibitory role of SIRT4 on IMM and OMM fusion and are consistent with the impact a greater IMM CSA has in decreasing the diffusion of substrates and electron transfer [26,35] and increasing the distribution of OXPHOS [25].

These observations promote the role SIRT4 has been shown to have in the regulation of fatty acid oxidation [45] and were further confirmed within our HSKMC model through high-resolution respirometry (HRR),

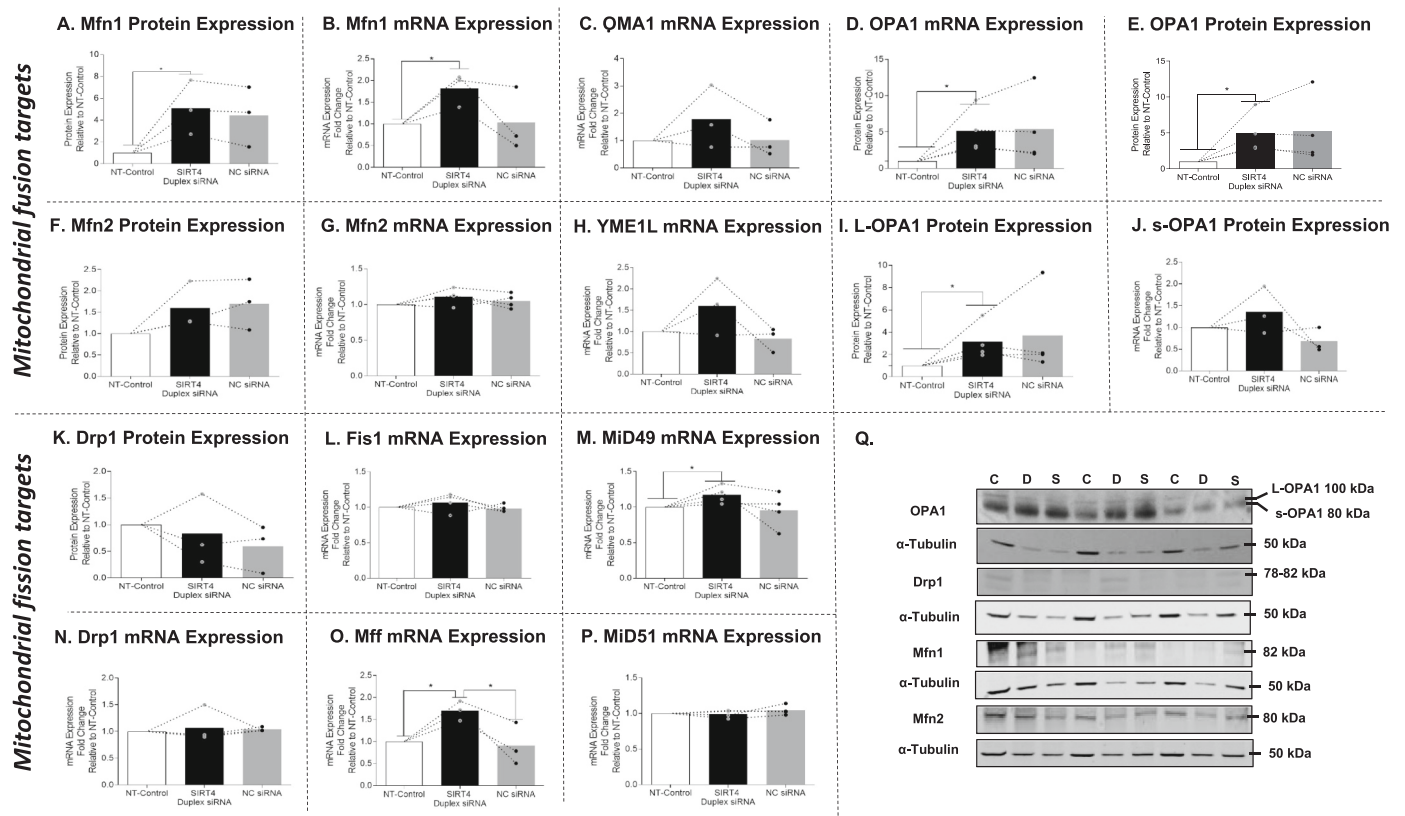


Fig. 4. SIRT4's regulation of other mitochondrial fusion and fission targets. Mitochondrial fusion targets: total Mfn1 (A), Mfn2 (F), OPA1 (E), L-OPA1 (F) and (J) s-OPA1 protein expression in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes quantified by western blot ($n = 3$). Total Mfn1 (B), Mfn2 (G), OPA1 (D), OMA1 (C) and YME1L (H) mRNA expression in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes quantified by RT-qPCR ($n = 4$). Mitochondrial fission targets: total Drp1 protein expression (K) in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes quantified by western blot ($n = 3$). Total Drp1 (N), Fis1 (L), Mff (O), MiD49 (M) and MiD51 (P) mRNA expression in NT-Control, SIRT4 Duplex (75 nM) siRNA and NC-siRNA (75 nM) HSkMC myotubes quantified by RT-qPCR ($n = 4$). Protein expression was normalised against α -Tubulin expression of same sample. Gene expression was normalised against GAPDH mRNA expression of same sample. (Q) Blots of all targets with respective loading controls and molecular weight in kilodaltons (kDa). White bars, NT-Control; black bars, SIRT4 Duplex (75 nM) siRNA; grey bars, NC-siRNA (75 nM). Individual response represented by dots; SIRT4 Duplex (75 nM) siRNA (grey), NC-siRNA (75 nM) (black). Data are expressed relative to NT-Control as mean $\pm$ SD, * significant difference between groups, $p < 0.05$.

noting how SIRT4 knockdown increased ADP-mediated oxidative phosphorylation (OXPHOS) (state 4) respiration, indicative of an increase in fatty acid oxidation [46]. Additionally, the reduction in LEAK (State 3) respiration with SIRT4 knockdown promote the regulatory role SIRT4 has on governance of mitochondrial efficiency via the control of ADP-ATP translocation across the inner mitochondrial membrane through the regulation of Adenosine Nucleotide Translocase 1 (ANT1), the most abundant protein on the IMM [32,47,48]. These changes were further supported by the reduction in ANT1 protein expression with SIRT4 knockdown.

In line with the increase in function, the promotion in fatty acid oxidation was further supported by increases in the translational expression of regulators of skeletal muscle fatty acid oxidation. Although we found no change in pAMPK^{Thr172}/AMPK protein expression (see Supplementary Data, Fig. 9. C) with SIRT4 knockdown, we did observe an increase in pACC^{Ser79}, which is often used as a surrogate for AMPK activity. These adaptations in protein expression, in line with improvements in oxidative phosphorylation, exhibit the inhibitory role SIRT4 has on the oxidation of fatty acids and how a reduction in its expression acts to improve fatty acid utilization.

Previous evidence by Nasrin et al. [45] demonstrated how SIRT4 inhibition acted to increase fatty acid oxidation, as measured by ³H-palmitoyl-L-carnitine, in primary mouse myotubes. While that by Laurent and colleagues [49], emphasized how overexpression of SIRT4 repressed fatty acid oxidation and further stimulated lipogenesis using similar methods. Although using a different technique, our data is in

support of this work, identifying the regulatory role of SIRT4 on overall cellular oxidation of fatty acids [45,49]. However, this is the first evidence to signify this role within the HSkMC model. Additionally, this is the first instance to identify SIRT4 as a negative regulator of OPA1, aiding in the governance of the mitochondrial phenotype. Knockdown of SIRT4 increases the expression of OPA1 and Mfn1, suggestive of an increase in mitochondrial fusion as depicted in Fig. 5. Successive MiNA analysis, indicates how this improvement in mitochondrial fusion, increases mitochondrial elongation following SIRT4 knockdown. An increase in mitochondrial elongation has previously been proven to increase IMM CSA and thereby increase the potential for regulators of oxidative phosphorylation [39,40,42]. Here we show that this increase in elongation results in a significant increase in OXPHOS, linked to a decrease in LEAK respiration via SIRT4's direct deacetylation of ANT1. This increase in OXPHOS is further supported by increases in regulators of fatty acid metabolism; pACC^{Ser79}, CPT1b and PGC-1 α . As such, inhibition of SIRT4 increases mitochondrial elongation, improving the metabolism of fatty acids, a mechanism which may be integral in the combat against obesity-related disease.

In conclusion, our results suggest SIRT4 has the capability to be a novel therapeutic to counteract many physiological changes associated with metabolic disease progression such as diet-induced obesity, while also acting as an exercise mimetic to increase exercise tolerance. More evidence is necessary to fully elucidate this prediction through in-vivo trials, but in-vitro evidence proves a governing role of SIRT4 over mitochondrial fusion and subsequently overall cellular metabolism.

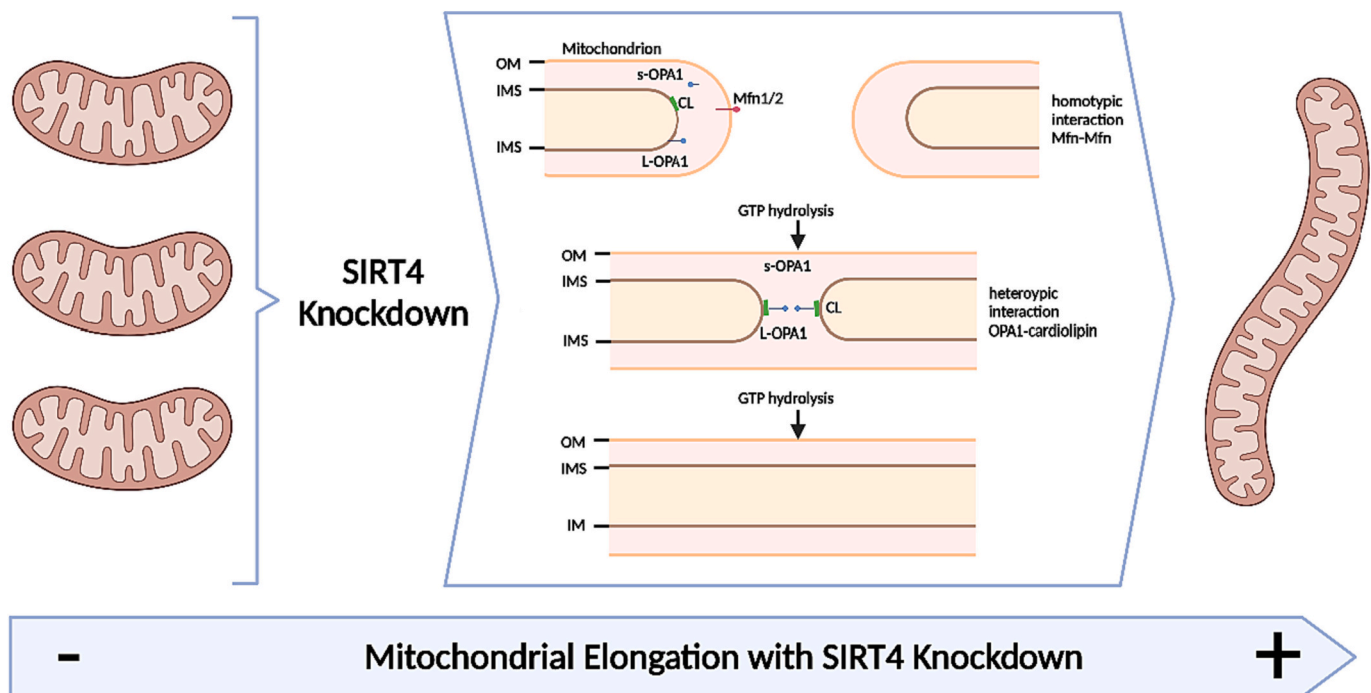


Fig. 5. Schematic representation of the impact SIRT4 knockdown has on mitochondrial elongation. Created with [BioRender.com](https://www.biorender.com).

Author contributions

JN and DOG conceived and designed the study and wrote the manuscript. JN performed all study experiments, along with data analyses. KR provided critical direction to JN in the HSKMC KO optimisation. FOS supervised and provided important advice to JN for all microscopy work. DOG provided essential guidance in data interpretation and concepts. DOG, KR and FOS supervised and provided necessary guidance to JN in the manuscript preparation and study design. All authors read and provided comments on the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary information can be found here: <https://figshare.com/s/9766380a01fae1006224>, DOI: <https://doi.org/10.6084/m9.figshare.22652722>.

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