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ORIGINAL RESEARCH ARTICLE

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MicroRNA-382 silencing induces a mitonuclear protein imbalance and activates the mitochondrial unfolded protein response in muscle cells

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Abstract

Proper mitochondrial function plays a central role in cellular metabolism. Various diseases as well as aging are associated with diminished mitochondrial function. Previously, we identified 19 miRNAs putatively involved in the regulation of mitochondrial metabolism in skeletal muscle, a highly metabolically active tissue. In the current study, these 19 miRNAs were individually silenced in C2C12 myotubes using antisense oligonucleotides, followed by measurement of the expression of 27 genes known to play a major role in regulating mitochondrial metabolism. Based on the outcomes, we then focused on miR-382-5p and identified pathways affected by its silencing using microarrays, investigated protein expression, and studied cellular respiration. Silencing of miRNA-382-5p significantly increased the expression of several genes involved in mitochondrial dynamics and biogenesis. Conventional microarray analysis in C2C12 myotubes silenced for miRNA-382-5p revealed a collective downregulation of mitochondrial ribosomal proteins and respiratory chain proteins. This effect was accompanied by an imbalance between mitochondrial proteins encoded by the nuclear and mitochondrial DNA (1.35-fold, $p < 0.01$) and an induction of HSP60 protein (1.31-fold, $p < 0.05$), indicating activation of the mitochondrial unfolded protein response (mtUPR). Furthermore, silencing of miR-382-5p reduced basal oxygen consumption rate by 14% ($p < 0.05$) without affecting mitochondrial content, pointing towards a more efficient mitochondrial function as a result of improved mitochondrial quality control. Taken together, silencing of miR-382-5p induces a mitonuclear protein imbalance and activates the mtUPR in skeletal muscle, a phenomenon that was previously associated with improved longevity.

KEYWORDS
microRNA, mitochondria, protein stress, skeletal muscle

1 | INTRODUCTION

Mitochondria play a central role in the regulation of cellular metabolism. Various pathologies as well as aging have been associated with mitochondrial dysfunction (Dominic et al., 2014; Johannsen & Ravussin, 2009; Lin & Beal, 2006). For example, over the last decade an extensive amount of research has shown that skeletal muscle mitochondrial abundance, size, and oxidative capacity are reduced in metabolic diseases, such as type 2 diabetes mellitus (T2DM; Coen et al., 2013; Kelley, He, Menshikova, & Ritov, 2002; Kim, Hickner, Cortright, Dohm, & Houmard, 2000; Lefort et al., 2010).

However, in recent years it has become increasingly clear that mitochondrial function is complex and depends on far more factors than merely mitochondrial abundance and OxPhos enzyme capacity (reviewed in Dahlmans, Houzelle, Schrauwen, & Hoeks, 2016; Quiros, Mottis, & Auwerx, 2016). Although mitochondria were classically seen as individual organelles, it is now evident that mitochondria form a dynamic network that is constantly being remodeled by the removal of damaged mitochondria (fission) and fusion of healthy mitochondria (Smirnova, Shurland, Ryazantsev, & van der Bliek, 1998), to maintain proper mitochondrial morphology and function.

Furthermore, additional mitochondrial quality control mechanisms have been identified, which are crucial for efficient mitochondrial function and also play a fundamental role in health and disease (Haelterman et al., 2014; Imatoh et al., 2009; Quiros et al., 2016). An important mitochondrial quality control mechanism is the mitochondrial unfolded protein response (Zhao et al., 2002; mtUPR), an evolutionary conserved protein quality control mechanism that has been shown to be deregulated in Parkinson's disease (reviewed in Haelterman et al., 2014) and has also been linked to T2DM (Henstridge et al., 2014). The main role of the mtUPR is to resolve proteotoxic stress, that is, the accumulation of harmful protein aggregates consisting of misfolded and damaged proteins, through the actions of chaperones and proteases (Haynes, Petrova, Benedetti, Yang, & Ron, 2007), which assist in the folding of misfolded or unfolded proteins and the degradation of misfolded proteins (reviewed in Haynes et al., 2007; Jovaisaite & Auwerx, 2015).

MicroRNAs (miRNAs) are short oligonucleotides of 20–22 nucleotides in length that posttranscriptionally regulate protein translation by base pairing with protein-coding transcripts, which have been shown to regulate numerous important cellular processes (Bartel, 2004, 2009; Mohamed, Hajira, Pardo, & Boriek, 2014; Zhang et al., 2013). Interestingly, during the last decade miRNAs have been reported to not only be present in mitochondria, but also to regulate various aspects of mitochondrial function (Barrey et al., 2011; Bian et al., 2010; El Azzouzi et al., 2013; Li et al., 2014; Mohamed et al., 2014; Sripada, Tomar, & Singh, 2012). In this context, we recently performed an unbiased, hypothesis-free, high throughput miRNA silencing screen in C2C12 muscle cells, and identified 19 miRNAs, which upon silencing, had a positive impact on mitochondrial metabolism (Dahlmans et al., 2017).

Here we extend these findings and measured the expression of 27 genes involved in different aspects of mitochondrial function upon individual silencing of these 19 candidate miRNAs in fully differentiated C2C12 myotubes. Based on the results, we singled out miR-382-5p and show that silencing of miR-382-5p leads to a collective downregulation of the transcripts encoding for the mitochondrial ribosomal proteins, induces a mitonuclear protein imbalance and activates the mtUPR.

2 | METHODS

2.1 | Cell culture and transfection

Myoblasts were seeded in 12-well plates at 100,000 cells/cm^2 in growth media (Dulbecco's modified Eagle medium 4.5 g/l glucose, with 10% fetal bovine serum, 2% HEPES, and 1% nonessential amino acids) and reached full confluence in 24 hr. Once confluence was reached, differentiation was initiated by exchanging the growth media for differentiation media (Dulbecco's modified Eagle medium 4.5 g/l glucose, with 2% horse serum, 2% HEPES, and 1% nonessential amino acids). The media was changed regularly and mature myotubes were obtained after 5 days of differentiation.

Transfections were either conducted at Day 5 or 6 of differentiation. To this end, the cell media was changed to 400 µl fresh differentiation media and the cells were transfected with miRNA inhibitors using lipofectamine RNAimax, according to the manufacturers protocol, reaching a total volume of 500 µl per well. The final concentration for transfection (50 nM) was previously established in optimization experiments using increasing concentrations of a miRNA inhibitor against the muscle-specific miRNA-206 (Dahlmans et al., 2017). Assays were always performed at Day 7 of differentiation, either 24 or 48 hr after the transfection.

2.2 | Individual miRNA inhibitors

Individual mouse miRCURY LNA™ microRNA inhibitors were purchased from (Exiqon A/S (Vedbaek, Denmark). MiRCURY LNA™ microRNA inhibitor control/negative control A (Exiqon A/S, Vedbaek, Denmark) was used as negative control. This control (sequence: TAACACGTCTATACGCCCA) does not target any known mature miRNA of the online miRbase database.

2.3 | Microarray analysis

Raw microarray data derived from Affymetrix MoGene 1.1 ST arrays (Santa Clara, CA) were collected and processed per data set into normalized gene expression values using RMAExpress (Bolstad, Irizarry, Astrand, & Speed, 2003) as previously described (Sanderson et al., 2009). Volcano plot was created by using GraphPad v6. Heatmaps were created using GENE-E software (www.broadinstitute.org/cancer/software/GENE-E), which transformed log2-scale gene expression values in each row into z-scores and subsequently converted these to heatmap colors. Raw microarray data were deposited in the GEO archive (GSE116786). Gene Set Enrichment Analysis (GSEA) was performed using the GSEA tool (Subramanian et al., 2005), which was

obtained from Broad Institute (http://software.broadinstitute.org/gsea/index.jsp), with mitochondrial gene sets defined previously (Andreux et al., 2014). Gene sets with a false discovery rate below 0.25 were considered significant.

2.4 | Protein extraction and immunoblot analysis

Cells were washed with 1 ml cold 1xphosphate-buffered saline (PBS) and scraped in 40 µl bio-plex cell lysis buffer (Biorad), and processed according to the manufacturers guidelines. OXPHOS proteins were detected using a cocktail of five monoclonal antibodies directed against structural components of the different OXPHOS complexes (MS601, MitoSciences, Eugene, OR), as previously described (Hoeks et al., 2010). Furthermore, VDAC (sc-8828, Santa Cruz Biotech, Heidelberg, Germany), HSP60 (Sc-1052 Santa Cruz Biotech, Heidelberg, Germany), and HSP90 (610418, BD Biosciences, Breda, Netherlands) were detected using separate antibodies from different manufacturers.

2.5 | RNA extraction, cDNA synthesis, and quantitative RT-PCR analysis

Cells were washed with 1 ml ice-cold 1xPBS, harvested in 700 µl Trizol reagent (Invitrogen, Breda, The Netherlands) and stored at −80°C for later use. RNA was isolated using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). RNA quantity and quality were assessed spectrophotometrically (ND-1000, NanoDrop Technologies, Wilmington) and with 6000 Nano chips (Bioanalyzer2100; Agilent, Amstelveen, The Netherlands). cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). A SensiMix SYBR Hi-ROX kit (Bioline, London, United Kingdom) was used for PCR amplification according to manufacturer's instructions. The following protocol was used for every amplification: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, comparing the expression of miRNA silenced C2C12 myotubes to C2C12 myotube transfected with negative control A, using the geometrical mean of 36B4 and B2M gene expression as a reference. Heatmaps were generated from the average relative expression of three independent experiments, unless stated otherwise, using R (R Foundation for Statistical Computing, Vienna, Austria).

For the determination of mitochondrial DNA copy number, total DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, The Netherlands). Total DNA was quantified and the integrity was checked by spectrophotometry using the Nanodrop. Relative amounts of nuclear and mtDNA were quantified by quantitative polymerase chain reaction (qPCR), in which nuclear DNA was represented by the *UCP2* gene and mitochondrial DNA by the *COX2* gene (Table 1). qPCR was performed using HOT FIREPol DNA Polymerase (Solis Biodyne, Tartu, Estonia) in the ABI Prism 7900HT Real-Time PCR system (Applied Biosystems) with the following protocol: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s followed by 60°C for 1 min. MtDNA copy number was calculated with the following formula: 2ΔCt (ΔCt = CtCOX2 − CtUCP2).

2.6 | Cellular respiration in C2C12 myotubes

Oxygen consumption rate (OCR) was measured using the Seahorse XF96 equipment (Seahorse bioscience Inc., North Billerica, MA). On the day of the assay, the cell media was exchanged by 175 µl XF media, according the manufacturer's guidelines. Cells were placed at 37°C with ambient CO_2 concentrations for 45 min. Subsequently, the assay was started and OCRs were measured, three consecutive times with 3-min intervals. First at basal conditions to determine routine respiration, after an oligomycin injection to determine oligomycin-induced leak respiration, after an FCCP injection to measure maximal respiration and finally after an injection containing both antamycinA and rotenone, to determine nonmitochondrial respiration. After the assay, cells were washed twice with PBS and stored at −20°C for protein determination. Total protein concentrations were determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit (ThermoFisher, Bleiswijk, Netherlands) according to manufacturer's guidelines.

2.7 | Citrate synthase activity assays

C2C12 myotubes were washed in ice-cold PBS and scraped in 50 µl SET buffer 48 hr posttransfection. Next, protein concentrations were determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit and the samples were diluted in SET buffer to a concentration of 1 µg/µl, aliquoted and stored at −80°C. Briefly, for citrate synthase activity measurement, 250 µl of reaction buffer (Tris-base [100 mM], DTNB [100 µM], acetyl-CoA [50 µM], pH 8.0) was added to 5 µl sample (1 µg/µl). To initiate the reaction, 5 µl oxaloacetic acid (25 mM) was added and the change in absorbance at 412 nm was measured for 20 min at 37°C, with three readings per minute.

2.8 | Statistical analyses

Reported data are expressed as means ± standard error of the mean (*SEM*) where indicated. Differences in gene expression, protein abundance, respiration, and citrate synthase activity were assessed using Student's *t* tests. Array data was analyzed using GSEA software (Subramanian et al., 2005). Several gene sets were tested and the *q* value of the false discovery rate control inferior to 0.25 was used as a significance threshold.

3 | RESULTS

3.1 | miRNA-382-5p silencing induces changes in genes involved in mitochondrial dynamics and biogenesis in C2C12 myotubes

To investigate the role of previously identified (Dahlmans et al., 2017) miRNAs in the regulation of skeletal muscle mitochondrial function, we measured the expression of 27 genes involved in different aspects of mitochondrial function, such as mitochondrial biogenesis, metabolism, dynamics, and quality control (Table 1) upon individual silencing (24 hr and 48 hr) of these 19 candidate miRNAs.

TABLE 1 Primer overview. All primer oligonucleotide sequences are shown from 5′ to 3′. Genes with an asterisk represent primer pairs used for mitochondrial DNA copy number experiments

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
ClpP	TGTTGCGGGAACGCATCGTGT	TAGATGGCCAGGCCCGCAGTT
HSP60	GCTGTAGCTGTTACAATGGGG	TGACTTTGCAACAGTGACCC
PPARD	GCGGACCTGGGGATTAATGG	AGATGGACTGCCTTTACCGTG
CytC-1	ACGCTGACAGCATCACCTTT	GCTTCCAGGTGCAAGTGCT
COX1	CACTACCAGTGCTAGCCGCA	TCCTGGGAGGATAAGAATATAAACTTCT
mtATP8	CATTCCCACTGGCACCTTCA	GTTGGGGTAATGAATGAGGCAAA
mtCox2	AACCGAGTCGTTCTGCCAAT	CTAGGGAGGGGACTGCTCAT
SDHA	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA
SOD1	AGGCTGTACCAGTGCAGGAC	TTGTTTCTCATGGACCACCA
SOD2	GGCCAAGGGAGATGTTACAA	GAACCTTGGACTCCCACAGA
PGC1a	TGTCTCTGTGAGGACCGCTA	ACAGCTTTCTGGGTGGATTG
SIRT1	GTCTCCTGTGGGATTCCTGA	ACACAGAGACGGCTGGAACT
NRF1	CGGAGTGACCCAAACTGAAC	TGCCGTGGAGTTGAGGATGT
NRF2	CTGGATCCAGACCGAAGCTT	GATGACCACCTCGACCGTTT
FIS1	ATTTGAATATGCCTGGTGCC	GCTGTTCCTCTTTGCTCCCT
Acox1	CCGTCGAGAAATCGAGAACTTG	ATTGAGGCCAACAGGTTCCA
Acadl	TCTTGCGATCAGCTCTTTCA	GGTACATGTGGGAGTACCCG
PGC1b	ATCTCTCTGACACGCAGGGT	TCTTCGTAAGCGCAGCCAA
Cpt1b	GACCATAGAGGCACTTCTCAGCATGG	GCAGCAGCTTCAGGGTTTGT
OPA1	TGACAAGCATTACAGGAAGGTGTCAGA	TCATCTCGCCGGACCCTCTCG
TFAM	AAGTGTTTTTCCAGCATGGG	GGCTGCAATTTTCCTAACCA
MFN1	AGGGGACCGATGGAGATAAAG	AAGAGGGCACATTTTGCTTTG
MFN2	ACGTCAAAGGGTACCTGTCCA	CAATCCCAGATGGCAGAACTT
NDUFS1	GCCAGTTGTGCGAACATATC	GGAACTACTCGGTGGGCTC
UCP2	ACCAAGGGCTCAGAGCATGCA	TGGCTTTCAGGAGAGTATCTTTG
ATP5g1	CAGGAAGGCTGCTTAGATGG	CACTGCTCATTTCTCCAGCTC
COX4	GATCAGCGTAAGTGGGGAAA	GAGCCTGATTGGCAAGAGAG
36b4	AGATTCGGGATATGCTGTTGG	AAAGCCTGGAAGAAGGAGGTC
B2m	ATGGGAAGCCGAACATACTG	CAGTCTCAGTGGGGGTGAAT
*COX2**	TTTTCAGGCTTCACCCTAGATGA	GAAGAATGTTATGTTTACTCCTACGAATATG
*UCP2**	GCGTTCTGGGTACCATCCTAAC	GCGACCAGCCCATTGTAGA

The analysis revealed distinct expression patterns as visualized in a clustered heatmap (Figure 1a,b) clearly demonstrating the different effects on the expression of the measured mitochondria-associated genes upon silencing of the miRNAs. One of the miRNAs that upon silencing gave an interesting mitochondria-associated gene expression pattern was miR-382-5p. Specifically, 24 hr of miR-382-5p silencing led to a collective upregulation of the mRNAs of Mitofusin 1 (MFN1), Mitofusin 2 (MFN2), and Optic atrophy 1 (OPA1; $p = 0.009$), all major factors involved in mitochondrial dynamics (Figure 1a). Furthermore, uncoupling protein 2 (UCP2) gene expression was increased by 1.7-fold at 24 hr posttransfection. In addition, 48 hr posttransfection, we observed that miR-382-5p silencing resulted in a robust induction of the transcript levels of the peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) and sirtuin1 (SIRT1), key genes involved in the regulation of mitochondrial biogenesis and metabolism (Figure 1b). Silencing of miR-382-5p was confirmed by RT-qPCR (Supporting Information Supplementary Figure 1).

These results suggest an important role for miR-382-5p in the control of mitochondrial function.

3.2 | miRNA-382-5p silencing collectively downregulates mitochondrial ribosomal subunit gene expression

To gain more insight in the pathways which are affected by silencing miR-382-5p silencing, we next conducted microarray analyses in C2C12

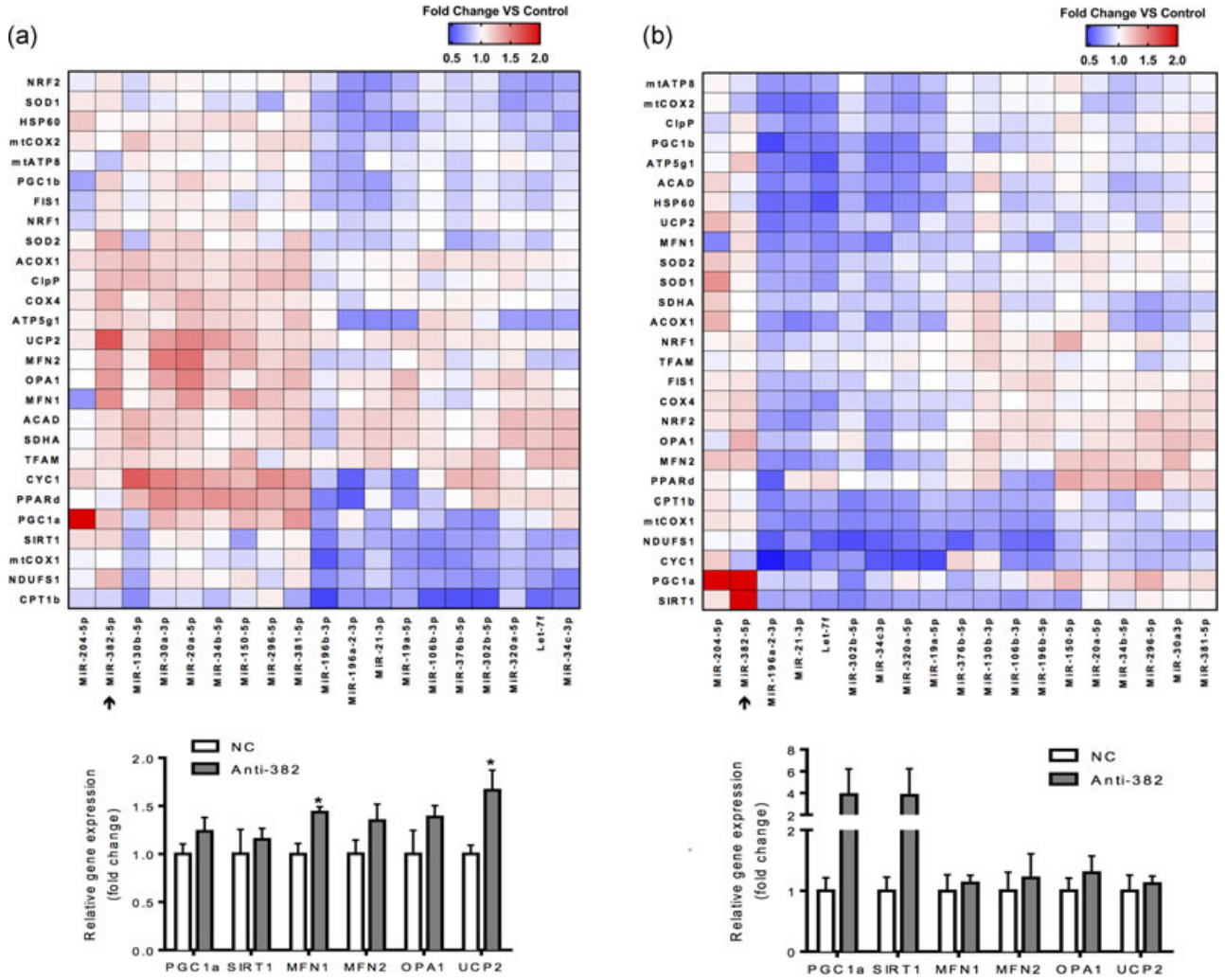

FIGURE 1 miR-382-5p silencing induces genes related to mitochondrial biogenesis and dynamics. Hierarchical clustering of the expression of 27 genes involved in different aspects of mitochondrial function, average expression of three independent experiments, (a) 24 and (b) 48 hr after individually transfecting our 19 candidate miRNA inhibitors, with blue and red representing down and upregulation, respectively. White represents no change. The graph shows the average ± *SEM* fold change of three independent experiments ($n = 3$) relative to negative control A, with * representing a $p < 0.05$ [Color figure can be viewed at wileyonlinelibrary.com]

myotubes after 24 hr of miR-382-5p silencing (Figure 2). Figure 2a shows a volcano plot visualizing the up- and downregulated transcripts in response to miR-382-5p silencing. Subsequently, gene set enrichment analysis revealed that 12 mitochondrial pathways were substantially downregulated, all with a FDR *q*-value below 0.25 and a normalized enrichment score below −1.5 (Figure 2b). Relative to other genome-wide enriched pathways, the mitochondrial oxidative phosphorylation gene set was also one of the most affected pathways (Supporting Information Supplemental Figure 2). Besides downregulating gene sets related to the mitochondrial electron transport chain (ETC), miR-382-5p silencing also downregulated gene sets related to the mitochondrial ribosome (Figure 2c,d). This latter finding caught our attention, since previous research by our research group showed that downregulation of mitochondrial protein translation as a result of decreased activity of the mitochondrial ribosomal machinery resulted in an imbalance between mitochondrial proteins encoded by the nuclear DNA and mitochondrial proteins encoded by the mitochondrial DNA (Houtkooper et al., 2013). Importantly, this so-called mitonuclear protein imbalance activated the mtUPR, which improved mitochondrial protein quality control and lifespan in *Caenorhabditis elegan* s (Houtkooper et al., 2013). Furthermore, we previously reported that silencing of miR-382-5p led to a robust induction of the promoter activity of the mitochondrial chaperone heat shock protein 60 (HSP60), a marker for mtUPR activity, both in C2C12 myoblasts (2.2-fold) and in C2C12 myotubes (1.9-fold; Dahlmans et al., 2017).

3.3 | miRNA-382-5p silencing induces a mitonuclear protein imbalance

To test if the collective downregulation of genes encoding mitochondrial ribosomal protein subunits upon miR-382-5p silencing was indeed accompanied by a mitonuclear protein imbalance, we measured protein levels of Cytochrome C Oxidase I (MTCO1), a mitochondrial protein encoded by the mitochondrial DNA, and

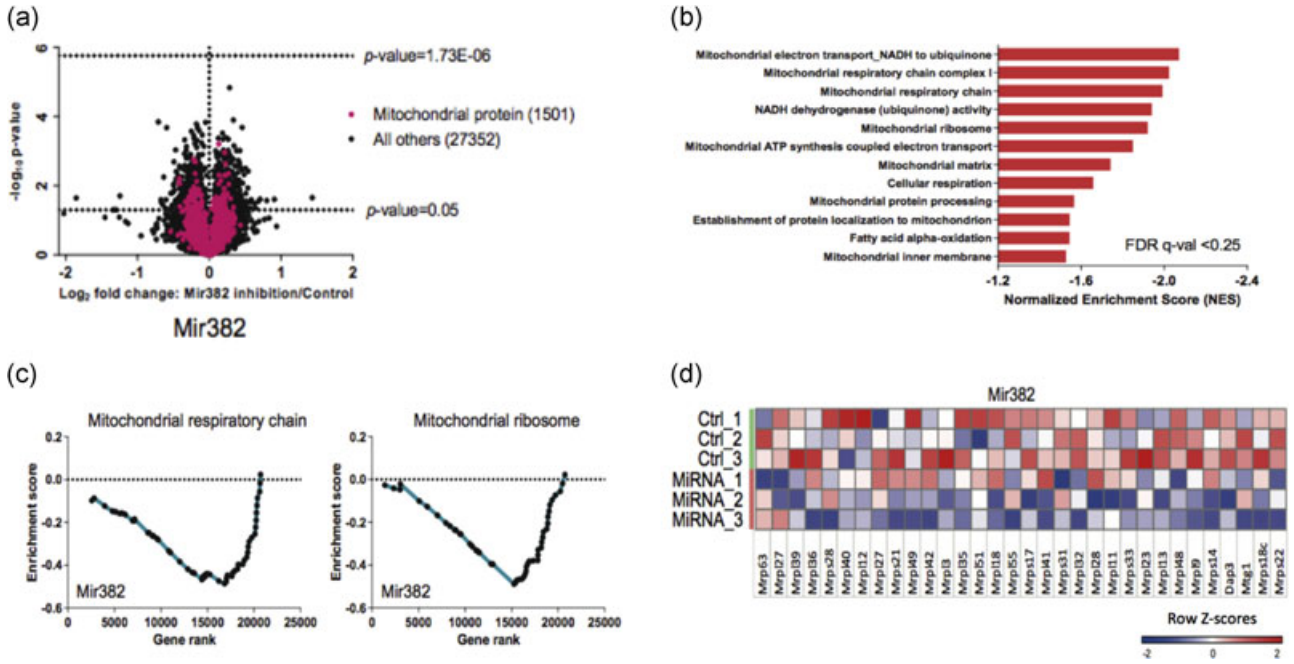

FIGURE 2 Microarray analysis of MiR-382-5p silencing in C2C12 myotubes. (a–d) Effects of MiR-382-5p silencing on gene expression in C2C12 myotubes 24 hr posttransfection. (a) Volcano plot of microarray data set, with mitochondrial gene set (GO:0005739) represented in red. (b) Overview of mitochondrial pathways that changed significantly with an FDR *q*-value lower than 0.25. (c) Negative enrichment scores for mitochondrial respiratory chain (GO:0005746, FDR *q*-value = 0.006) and mitochondrial ribosomal subunits (GO:0005761, FDR *q*-value = 0.0099) indicates that miR-382-5p silencing results in a downregulation of respiratory complexes and mitochondrial protein processing. (d) Heatmap displaying Row Z-scores of genes encoding mitochondrial ribosomal protein subunits. Downregulation of mitochondrial ribosomal proteins points towards a mitonuclear protein imbalance [Color figure can be viewed at wileyonlinelibrary.com]

succinate dehydrogenase subunit B (SDHB), a nuclear-encoded mitochondrial protein, over the course of 48 hr. Since miRNAs are known to be able to affect protein translation in relatively short timeframes, we measured the mitonuclear protein balance at 6, 12, 18, 24, and 48 hr posttransfection to make sure that we did not miss any transient effects. Indeed, 18 hr after silencing miR-382-5p in C2C12 myotubes, the ratio between these 2 proteins tended to increase compared with negative control A transfected cells (1.21-fold, $p = 0.07$), which reached statistical significance after 24 (1.25-fold, $p = 0.02$) and 48 hr (1.35-fold, $p = 0.0004$) of silencing (Figure 3), suggesting the development of a mitonuclear protein imbalance.

3.4 | miRNA-382-5p silencing activates the mtUPR and reduces basal oxygen consumption

To determine if the mitonuclear protein imbalance induced by miR-382-5p silencing was associated with activation of the mtUPR, we quantified the mitochondrial chaperone heat shock protein 60 (HSP60). At 6 and 18 hr posttransfection, small inductions of HSP60 were observed. However, in accordance with the mitonuclear protein imbalance, the level of HSP60 protein expression was highest 48 hr posttransfection compared with C2C12 myotubes transfected with negative control A (1.31-fold, $p = 0.002$), indicative of the activation of the mtUPR (Figure 4).

A mitonuclear protein imbalance and activation of the mtUPR has previously been associated with changes in mitochondrial respiratory capacity (Houtkooper et al., 2013). Therefore, we tested if silencing of miR-382-5p would affect basal OCRs. Interestingly, miR-382-5p silencing indeed reduced basal OCR by 14% in C2C12 myotubes, compared with negative control A transfected C2C12 myotubes ($p = 0.046$), 48 hr posttransfection (Figure 5a). However, this was not accompanied by a reduced citrate synthase activity or mitochondrial DNA copy number, indicating that basal skeletal muscle oxygen consumption was reduced without an apparent effect on total mitochondrial content (Figure 5b,c). Furthermore, the observed results in the mitochondrial phenotype are not likely to be caused by a change in fiber type composition upon miR-382-5p silencing (Supporting Information Supplementary Figure 3).

4 | DISCUSSION

We previously conducted an unbiased high throughput miRNA silencing screen in C2C12 myoblasts and myotubes to identify novel miRNAs involved in the regulation of skeletal muscle mitochondrial metabolism. To that end, we individually silenced > 700 miRNAs and investigated several functional parameters for mitochondrial function, and identified 19 miRNAs putatively involved in the regulation of muscle mitochondrial function (Dahlmans et al., 2017). In the current study, we extended these findings and determined the expression of 27 genes related to mitochondrial metabolism, after individual silencing of these 19 candidate miRNAs in C2C12

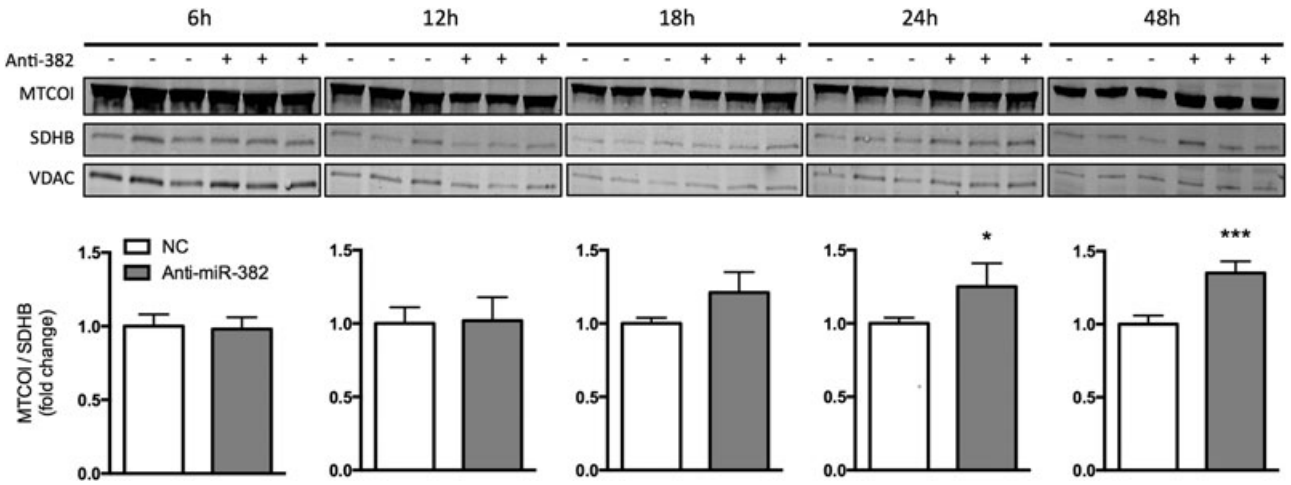

FIGURE 3 MiR-382-5p silencing induces a mitonuclear protein imbalance. Western blot analysis of C2C12 myotubes transfected with a miR-382-5p inhibitor, 6, 12, 18, 24, and 48 hr posttransfection ($n = 3$), showing increased levels of MTCO1 protein, a mitochondrial protein encoded by the mitochondrial DNA, whereas maintaining similar levels of SDHB protein, a mitochondrial protein encoded by the nuclear DNA. Graphs show the mitonuclear protein balance as a ratio of MTCO1 and SDHB protein levels, with *, and *** representing $p < 0.05$ and $p < 0.001$, respectively. Gels were loaded to achieve similar mitochondrial content, SDHB and VDAC

myotubes. Interestingly, silencing of miR-382-5p induced several genes involved in mitochondrial dynamics and biogenesis. Subsequent microarray analyses revealed a collective reduction in the mRNA levels of Mitochondrial Ribosomal Proteins (MRPs) upon miR-382-5p silencing, which in turn was accompanied by a mitonuclear protein imbalance and activation of the mtUPR.

In an attempt to gain more insight in the mechanisms by which the previously identified 19 candidate miRNAs contribute to the regulation of skeletal muscle mitochondrial function, we individually silenced these 19 candidate miRNAs in C2C12 myotubes and quantified 27 selected genes known to be involved in different aspects of mitochondrial function. Interestingly, miR-382-5p silencing resulted in an induction of several genes involved in mitochondrial dynamics (MFN-1, MFN-2, and OPA-1) and biogenesis (SIRT1 and PGC-1α) 24 hr posttransfection (Figure 1). To the best of our knowledge, miR-382-5p has not been linked to muscle mitochondrial metabolism before, which encouraged us to follow-up on this miRNA and study its role in the regulation of mitochondrial function in more detail.

Accordingly, we conducted conventional microarray analysis with subsequent gene set enrichment analysis. It was found that near-complete silencing of miR-382-5p in C2C12 myotubes downregulated several mitochondrial pathways (Figure 2b), including the expression of the gene set encoding for various MRPs. Interestingly, Mrps5 and other members of the MRP family have been identified as regulators of metabolism and longevity (Houtkooper et al., 2013). Specifically, it was shown that the gene expression of mitochondrial

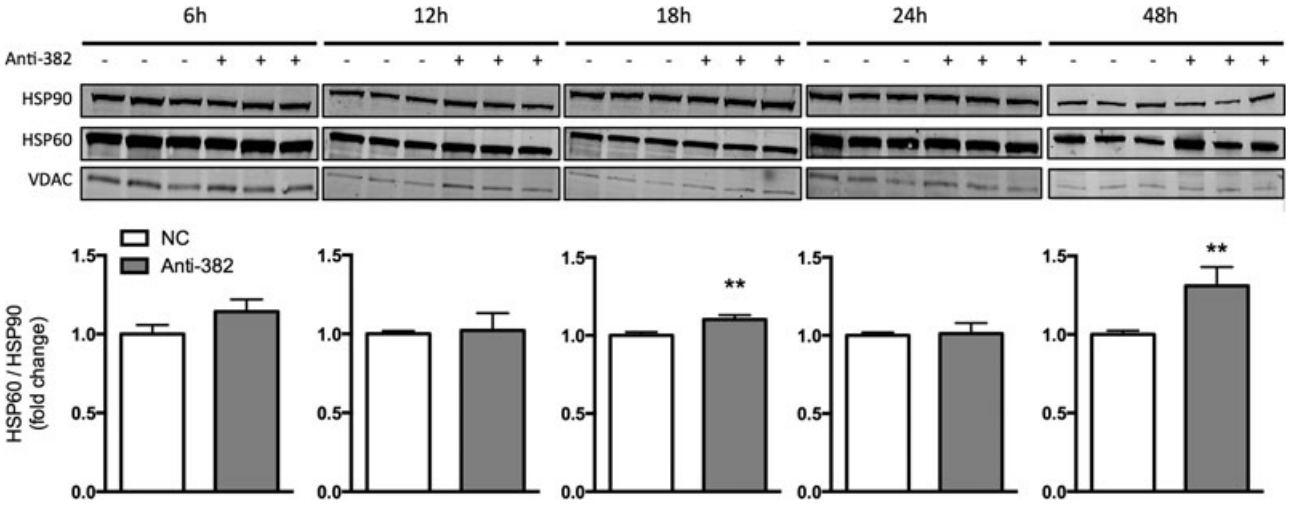

FIGURE 4 MiR-382-5p silencing activates the mtUPR. Western blot analysis of C2C12 myotubes transfected with a miR-382-5p inhibitor show increased levels of HSP60, 48 hr posttransfection ($n = 3$), without affecting the cytosolic HSP90. Graphs show mitochondrial chaperone heat shock protein 60 (HSP60), and heat shock protein 90 (a cytosolic chaperone) abundance, loaded for similar mitochondrial content (VDAC), with *, and ** representing $p < 0.05$ and $p < 0.01$, respectively

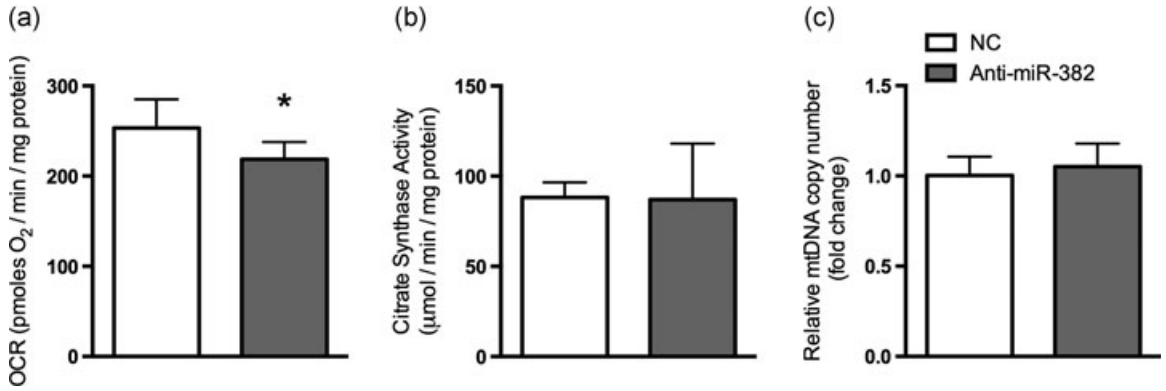

FIGURE 5 MiR-382-5p silencing in C2C12 myotubes reduces basal oxygen consumption without affecting mitochondrial content. (a) Basal oxygen consumption of the C2C12 myotubes after 48 hr of miR-382-5p silencing was significantly reduced ($n = 6$). (b,c) Enzymatic activity of citrate synthase ($n = 3$) and mitochondrial DNA copy number ($n = 3$) did not change in C2C12 myotubes after 48 hr of miR-382-5p silencing, with * representing $p < 0.05$

ribosomal protein S5 (Mrps5) and other members of the MRP family were negatively correlated with longevity in the BXD mouse reference population (Houtkooper et al., 2013). To further validate this finding, Mrps-5 was knocked down in *C. elegans* using RNAi-mediated knockdown, which resulted in a markedly increased lifespan and triggered a stoichiometric imbalance of mitochondrial components (i.e., "mitonuclear protein imbalance": Houtkooper et al., 2013). In addition, we recently highlighted in mammals that an induction of mitochondrial stress led to a collective downregulation of mitochondrial ribosomal proteins, indicating that the MRPs are a nodal point in the mitochondrial stress pathways (Quiros et al., 2017). All together, these results prompted us to investigate whether the collective downregulation of MRPs, which we observed upon silencing of miR-382-5p, was linked to an induction of a mitonuclear protein imbalance and activation of mitochondrial protein stress pathways.

To this end, we measured the stoichiometric balance of mitochondrial OXPHOS components over the course of a 48-hr timespan. We found that the collectively reduced expression of the MRP gene set upon miR-382-5p silencing was associated with the gradual development of a mitonuclear protein imbalance over time, which started to change 18 hr posttransfection, but was most pronounced 48 hr posttransfection (Figure 3). Strikingly, however, although MRP gene expression was reduced, we observed an increased MTCO1 protein abundance, a mitochondrial protein encoded by the mitochondrial DNA. Possibly, the disturbances in the mitonuclear protein balance generate a negative feedback regulation on the MRP gene expression. This notion is supported by our recent findings in plants, showing that the repression of mitochondrial translation induces a robust UPR^{mt}, which in turn triggers a response aimed to normalize the mitonuclear protein imbalance by repairing mitochondrial translation (via induction of the MRPs) and mitochondrial protein import/folding (Wang & Auwerx, 2017).

The mismatch between the nDNA- and mtDNA-encoded subunits of mitochondrial components is characteristic of a mitonuclear protein imbalance, and has been shown to cause unfolded protein stress, which subsequently activates mitochondrial protein quality control mechanisms (i.e., mtUPR; Houtkooper et al., 2013; Jovaisaite & Auwerx, 2015). The mtUPR assists in protein folding and degradation, and thereby protects against damaged proteins or protein aggregates. Moreover, in addition to a mitonuclear protein imbalance, the mtUPR is also activated upon mitochondrial biogenesis, mtDNA depletion (Yoneda et al., 2004), ETC subunit loss (Durieux, Wolff, & Dillin, 2011) and interference with mitochondrial architecture (Zhang et al., 2016), and protein import (Rainbolt, Atanassova, Genereux, & Wiseman, 2013) and translation (Houtkooper et al., 2013). Furthermore, previous reports demonstrated that activation of the mtUPR through *cco-1* (a nuclear encoded subunit of the ETC) and *spg-7* (a mitochondrial protein quality control protease) loss of function, activated the mtUPR and increased lifespan in *C. elegans* (Durieux et al., 2011; Wu et al., 2014; Yoneda et al., 2004). Therefore, we investigated the abundance of heat shock protein 60 (HSP60), a key protein chaperone of the mtUPR that resides in the mitochondrial matrix. Of note, 48 hr posttransfection when the mitonuclear protein imbalance was most pronounced, activation of the mtUPR was also observed (Figure 4).

Furthermore, it was reported that the Mrps5 silenced worms displayed a reduced OCR and lowered citrate synthase activity (Houtkooper et al., 2013). Although the worms used less oxygen and had lower citrate synthase activity, their longevity was improved, whereas the worms were more active and moved twice as much compared with the control worms (Houtkooper et al., 2013). In our study, we also observed reduced OCRs in miR-382-5p silenced C2C12 myotubes 48 hr posttransfection, albeit to a lesser extent. In contrast to the findings in worms, however, we did not observe reduced citrate synthase activity or reduced mitochondrial DNA copy number, two commonly used markers of mitochondrial content (Wang, Hiatt, Barstow, & Brass, 1999), indicating that the reduction of OCR is not due to loss of mitochondria.

Besides longevity, activation of the mtUPR may also have implications for metabolic diseases such as T2DM. Specifically, some evidence exists that members of the *mtUPR* gene set are deregulated

in both animal models for obesity/diabetes and human obese and type T2DM subjects (Bruce, Carey, Hawley, & Febbraio, 2003; Chung et al., 2008; Drew et al., 2014; Henstridge et al., 2014; Imatoh et al., 2009; Rong et al., 2007). However, most studies have been conducted in PBMCs or adipose tissue and relatively few studies have used skeletal muscle. Reports in skeletal muscle, however, do show that the mtUPR is impaired in mouse models for obesity/diabetes and that there is a close link between components of the mtUPR and mitochondrial function and morphology (Drew et al., 2014; Gariani et al., 2016; Henstridge et al., 2014; Rong et al., 2007; Zhang et al., 2016). Furthermore, reduced activity of heat shock proteins was not only observed in skeletal muscle of obese and T2DM human subjects compared with controls (Bruce et al., 2003; Chung et al., 2008), but also in plasma of T2DM subjects compared with lean controls (Imatoh et al., 2009). These animal and human studies hence suggest the importance of a functional mtUPR, and underscore the relevance of modulators of the mtUPR such as miR-382-5p.

In conclusion, we previously identified specific miRNAs as modulators of skeletal muscle mitochondrial metabolism. Here, we singled out miR-382-5p and showed that silencing of this miRNA leads to a collective downregulation of mitochondrial ribosomal protein expression, induces a mitonuclear protein imbalance, and activates the mtUPR, previously associated with improved longevity.

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CONFLICTS OF INTEREST

The authors have declared that there are no conflicts of interest.

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REFERENCES

Andreux, P. A., Mouchiroud, L., Wang, X., Jovaisaite, V., Mottis, A., Bichet, S., … Auwerx, J. (2014). A method to identify and validate mitochondrial modulators using mammalian cells and the worm *C. elegans*. *Scientific Reports*, *4*, 5285.

Barrey, E., Saint-Auret, G., Bonnamy, B., Damas, D., Boyer, O., & Gidrol, X. (2011). Pre-microRNA and mature microRNA in human mitochondria. *PLoS One*, *6*(5), e20220.

Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, *116*(2), 281–297.

Bartel, D. P. (2009). MicroRNAs: Target recognition and regulatory functions. *Cell*, *136*(2), 215–233.

Bian, Z., Li, L. M., Tang, R., Hou, D. X., Chen, X., Zhang, C. Y., & Zen, K. (2010). Identification of mouse liver mitochondria-associated miRNAs and their potential biological functions. *Cell Research*, *20*(9), 1076–1078.

Bolstad, B. M., Irizarry, R. A., Astrand, M., & Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*, *19*(2), 185–193.

Bruce, C. R., Carey, A. L., Hawley, J. A., & Febbraio, M. A. (2003). Intramuscular heat shock protein 72 and heme oxygenase-1 mRNA are reduced in patients with type 2 diabetes: Evidence that insulin resistance is associated with a disturbed antioxidant defense mechanism. *Diabetes*, *52*(9), 2338–2345.

Chung, J., Nguyen, A. K., Henstridge, D. C., Holmes, A. G., Chan, M. H. S., Mesa, J. L., … Febbraio, M. A. (2008). HSP72 protects against obesity-induced insulin resistance. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(5), 1739–1744.

Coen, P. M., Hames, K. C., Leachman, E. M., DeLany, J. P., Ritov, V. B., Menshikova, E. V., … Goodpaster, B. H. (2013). Reduced skeletal muscle oxidative capacity and elevated ceramide but not diacylglycerol content in severe obesity. *Obesity (Silver Spring)*, *21*(11), 2362–2371.

Dahlmans, D., Houzelle, A., Andreux, P., Jörgensen, J. A., Wang, X., de Windt, L. J., … Hoeks, J. (2017). An unbiased silencing screen in muscle cells identifies miR-320a, miR-150, miR-196b, and miR-34c as regulators of skeletal muscle mitochondrial metabolism. *Molecular Metabolism*, *6*(11), 1429–1442.

Dahlmans, D., Houzelle, A., Schrauwen, P., & Hoeks, J. (2016). Mitochondrial dynamics, quality control and miRNA regulation in skeletal muscle: Implications for obesity and related metabolic disease. *Clinical Science (London)*, *130*(11), 843–852.

Dominic, E. A., Ramezani, A., Anker, S. D., Verma, M., Mehta, N., & Rao, M. (2014). Mitochondrial cytopathies and cardiovascular disease. *Heart*, *100*(8), 611–618.

Drew, B. G., Ribas, V., Le, J. A., Henstridge, D. C., Phun, J., Zhou, Z., … Hevener, A. L. (2014). HSP72 is a mitochondrial stress sensor critical for Parkin action, oxidative metabolism, and insulin sensitivity in skeletal muscle. *Diabetes*, *63*(5), 1488–1505.

Durieux, J., Wolff, S., & Dillin, A. (2011). The cell-non-autonomous nature of electron transport chain-mediated longevity. *Cell*, *144*(1), 79–91.

El azzouzi, H., Leptidis, S., Dirkx, E., Hoeks, J., Van bree, B., Brand, K., … De windt, L. J. (2013). The hypoxia-inducible microRNA cluster miR-199a approximately 214 targets myocardial PPARdelta and impairs mitochondrial fatty acid oxidation. *Cell Metabolism*, *18*(3), 341–354.

Gariani, K., Menzies, K. J., Ryu, D., Wegner, C. J., Wang, X., Ropelle, E. R., … Auwerx, J. (2016). Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice. *Hepatology*, *63*(4), 1190–1204.

Haelterman, N. A., Yoon, W. H., Sandoval, H., Jaiswal, M., Shulman, J. M., & Bellen, H. J. (2014). A mitocentric view of Parkinson's disease. *Annual Review of Neuroscience*, *37*, 137–159.

Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y., & Ron, D. (2007). ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. *Developmental Cell*, *13*(4), 467–480.

Henstridge, D. C., Bruce, C. R., Drew, B. G., Tory, K., Kolonics, A., Estevez, E., … Febbraio, M. A. (2014). Activating HSP72 in rodent skeletal muscle increases mitochondrial number and oxidative capacity and decreases insulin resistance. *Diabetes*, *63*(6), 1881–1894.

Hoeks, J., van Herpen, N. A., Mensink, M., Moonen-Kornips, E., van Beurden, D., Hesselink, M. K. C., & Schrauwen, P. (2010). Prolonged fasting identifies skeletal muscle mitochondrial dysfunction as

consequence rather than cause of human insulin resistance. *Diabetes*, *59*(9), 2117–2125.

Houtkooper, R. H., Mouchiroud, L., Ryu, D., Moullan, N., Katsyuba, E., Knott, G., … Auwerx, J. (2013). Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature*, *497*(7450), 451–457.

Imatoh, T., Sugie, T., Miyazaki, M., Tanihara, S., Baba, M., Momose, Y., … Une, H. (2009). Is heat shock protein 60 associated with type 2 diabetes mellitus? *Diabetes Research and Clinical Practice*, *85*(2), 208–212.

Johannsen, D. L., & Ravussin, E. (2009). The role of mitochondria in health and disease. *Current Opinion in Pharmacology*, *9*(6), 780–786.

Jovaisaite, V., & Auwerx, J. (2015). The mitochondrial unfolded protein response-synchronizing genomes. *Current Opinion in Cell Biology*, *33*, 74–81.

Kelley, D. E., He, J., Menshikova, E. V., & Ritov, V. B. (2002). Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes*, *51*(10), 2944–2950.

Kim, J. Y., Hickner, R. C., Cortright, R. L., Dohm, G. L., & Houmard, J. A. (2000). Lipid oxidation is reduced in obese human skeletal muscle. *American Journal of Physiology, Endocrinology and Metabolism*, *279*(5), E1039–E1044.

Lefort, N., Glancy, B., Bowen, B., Willis, W. T., Bailowitz, Z., De Filippis, E. A., … Mandarino, L. J. (2010). Increased reactive oxygen species production and lower abundance of complex I subunits and carnitine palmitoyltransferase 1B protein despite normal mitochondrial respiration in insulin-resistant human skeletal muscle. *Diabetes*, *59*(10), 2444–2452.

Li, J., Li, Y., Jiao, J., Wang, J., Li, Y., Qin, D., & Li, P. (2014). Mitofusin 1 is negatively regulated by microRNA 140 in cardiomyocyte apoptosis. *Molecular and Cellular Biology*, *34*(10), 1788–1799.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), 787–795.

Mohamed, J. S., Hajira, A., Pardo, P. S., & Boriek, A. M. (2014). MicroRNA-149 inhibits PARP-2 and promotes mitochondrial biogenesis via SIRT-1/PGC-1alpha network in skeletal muscle. *Diabetes*, *63*(5), 1546–1559.

Quirós, P. M., Mottis, A., & Auwerx, J. (2016). Mitonuclear communication in homeostasis and stress. *Nature Reviews Molecular Cell Biology*, *17*(4), 213–226.

Quirós, P. M., Prado, M. A., Zamboni, N., D'amico, D., Williams, R. W., Finley, D., … Auwerx, J. (2017). Multi-omics analysis identifies ATF4 as a key regulator of the mitochondrial stress response in mammals. *Journal of Cell Biology*, *216*(7), 2027–2045.

Rainbolt, T. K., Atanassova, N., Genereux, J. C., & Wiseman, R. L. (2013). Stress-regulated translational attenuation adapts mitochondrial protein import through Tim17A degradation. *Cell Metabolism*, *18*(6), 908–919.

Rong, J. X., Qiu, Y., Hansen, M. K., Zhu, L., Zhang, V., Xie, M., … Ryan, T. E. (2007). Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone. *Diabetes*, *56*(7), 1751–1760.

Sanderson, L. M., Degenhardt, T., Koppen, A., Kalkhoven, E., Desvergne, B., Muller, M., & Kersten, S. (2009). Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) but not PPARalpha serves as a plasma free fatty acid sensor in liver. *Molecular and Cellular Biology*, *29*(23), 6257–6267.

Smirnova, E., Shurland, D. L., Ryazantsev, S. N., & van der Bliek, A. M. (1998). A human dynamin-related protein controls the distribution of mitochondria. *Journal of Cell Biology*, *143*(2), 351–358.

Sripada, L., Tomar, D., & Singh, R. (2012). Mitochondria: One of the destinations of miRNAs. *Mitochondrion*, *12*(6), 593–599.

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., … Mesirov, J. P. (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43), 15545–15550.

Wang, H., Hiatt, W. R., Barstow, T. J., & Brass, E. P. (1999). Relationships between muscle mitochondrial DNA content, mitochondrial enzyme activity and oxidative capacity in man: Alterations with disease. *European Journal of Applied Physiology and Occupational Physiology*, *80*(1), 22–27.

Wang, X., & Auwerx, J. (2017). Systems phytohormone responses to mitochondrial proteotoxic stress. *Molecular Cell*, *68*(3), 540–551. e545

Wu, Y., Williams, E. G., Dubuis, S., Mottis, A., Jovaisaite, V., Houten, S. M., … Aebersold, R. (2014). Multilayered genetic and omics dissection of mitochondrial activity in a mouse reference population. *Cell*, *158*(6), 1415–1430.

Yoneda, T., Benedetti, C., Urano, F., Clark, S. G., Harding, H. P., & Ron, D. (2004). Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *Journal of Cell Science*, *117*(Pt 18), 4055–4066.

Zhang, H., Ryu, D., Wu, Y., Gariani, K., Wang, X., Luan, P., … Auwerx, J. (2016). NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science*, *352*(6292), 1436–1443.

Zhang, Y., Yang, L., Gao, Y. F., Fan, Z. M., Cai, X. Y., Liu, M. Y., … Xia, Z. K. (2013). MicroRNA-106b induces mitochondrial dysfunction and insulin resistance in C2C12 myotubes by targeting mitofusin-2. *Molecular and Cellular Endocrinology*, *381*(1-2), 230–240.

Zhao, Q., Wang, J., Levichkin, I. V., Stasinopoulos, S., Ryan, M. T., & Hoogenraad, N. J. (2002). A mitochondrial specific stress response in mammalian cells. *EMBO Journal*, *21*(17), 4411–4419.

SUPPORTING INFORMATION

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