**Supplemental Information to the Manuscript by Zhang et al**

**Inventory of the Supplemental Information**

**Supplemental Table S1** is related to the experimental procedures section**:**
Statistics of Ago2 CLIP-seq data generated from C2C12 myoblasts and myotubes

**Supplemental Table S2** is related to the experimental procedures section**:**
Statistics of combined Ago2 CLIP-seq data and percentage distribution of Ago2 binding events on individual mitochondrial transcripts.

**Supplemental Table S3** is related to the experimental procedures section**:**
List of siRNAs and miRNAs used in the current study

**Supplemental Table S4** is related to the experimental procedures section**:**
List of PCR primers and probes used in the current study

**Figure S1** is related to main Figure 1**,** showing quantitative analysis of the mRNA levels of COX1 and ND1 during C2C12 myogenesis with 3 distinct primer sets against each transcript.

**Figure S2** is related to main Figure 2, showing the characterization and quantification of Ago2 in the mitochondria.

**Figure S3** is related to main Figure 3, illustrating the experimental scheme for miRACE and demonstrating the robustness of Ago2 CLIP-seq experiments on C2C12 myoblasts and myotubes.

**Figure S4** is related to main Figure 4, providing a series of supporting evidence for the direct effect of miR-1 in enhancing mitochondrial translation, rather than due to indirectly induced myogenic program.

**Figure S5** is related to main Figure 5, showing the evolutionary conservation of miR-1 target sites in ND1 and COX1 and luciferase reporter assays to characterize miR-1 target sites in the cytoplasm.

**Supplemental Experimental Procedures**

**References**

## Supplemental Tables

**Table S1.** Statistics of Ago2 CLIP-seq data generated from C2C12 myoblasts and myotubes

| | DM repeat 1 | | | GM repeat1 | | |
|---|---|---|---|---|---|---|
| Gene | Uniquely mapped on chrM | Mapped to nuclear genome | common | Uniquely mapped on chrM | Mapped to nuclear genome | common |
| ND6 | 472 | 0 | 634 | 117 | 0 | 150 |
| ND1 | 26164 | 18 | 3716 | 2319 | 8 | 216 |
| ND2 | 2137 | 1 | 1622 | 921 | 3 | 405 |
| COX1 | 5441 | 23 | 38653 | 1146 | 5 | 9132 |
| COX2 | 0 | 29 | 14940 | 0 | 5 | 4210 |
| ATP8 | 1 | 0 | 9870 | 0 | 0 | 1084 |
| ATP6 | 0 | 1 | 13065 | 0 | 0 | 2120 |
| COX3 | 0 | 1 | 23616 | 0 | 1 | 3366 |
| ND3 | 0 | 5 | 1845 | 0 | 3 | 511 |
| ND4L | 0 | 0 | 4593 | 0 | 0 | 1372 |
| ND4 | 2212 | 2 | 6071 | 886 | 0 | 3035 |
| ND5 | 12675 | 13 | 12084 | 2729 | 2 | 3144 |
| CYTB | 7116 | 7 | 7135 | 2888 | 1 | 2985 |
| | DM repeat 2 | | | GM repeat 2 | | |
| | Uniquely mapped on chrM | Mapped to nuclear genome | common | Uniquely mapped on chrM | Mapped to nuclear genome | common |
| ND6 | 550 | 3 | 725 | 168 | 0 | 232 |
| ND1 | 26863 | 18 | 3324 | 3363 | 15 | 418 |
| ND2 | 2154 | 7 | 1402 | 1360 | 0 | 813 |
| COX1 | 5934 | 27 | 39426 | 1567 | 5 | 14495 |
| COX2 | 1 | 19 | 14805 | 0 | 6 | 6663 |
| ATP8 | 0 | 1 | 10305 | 0 | 0 | 1717 |
| ATP6 | 0 | 2 | 12923 | 0 | 4 | 3435 |
| COX3 | 1 | 1 | 24172 | 0 | 2 | 5249 |
| ND3 | 0 | 10 | 1802 | 0 | 5 | 855 |
| ND4L | 0 | 0 | 4377 | 0 | 1 | 2289 |
| ND4 | 2137 | 1 | 6006 | 1314 | 0 | 5132 |
| ND5 | 12426 | 7 | 12322 | 4176 | 2 | 5184 |
| CYTB | 7241 | 8 | 7069 | 4259 | 6 | 5195 |

The number of Ago2 CLIP-seq tags uniquely mapped on chromosome M and to homologous nuclear region, common means multiply mapped on both.

**Table S2.** Statistics of combined Ago2 CLIP-seq data and percentage distribution of Ago2 binding events on individual mitochondrial transcripts.

| gene | total | | | sum |
|---|---|---|---|---|
| | Uniquely mapped on chrM | Mapped to nuclear genome | common | |
| ND6 | 1307 | 3 | 1741 | 3051 |
| ND1 | 58709 | 59 | 7674 | 66442 |
| ND2 | 6572 | 11 | 4242 | 10825 |
| COX1 | 14088 | 60 | 101706 | 115854 |
| COX2 | 1 | 59 | 40618 | 40678 |
| ATP8 | 1 | 1 | 22976 | 22978 |
| ATP6 | 0 | 7 | 31543 | 31550 |
| COX3 | 1 | 5 | 56403 | 56409 |
| ND3 | 0 | 23 | 5013 | 5036 |
| ND4L | 0 | 1 | 12631 | 12632 |
| ND4 | 6549 | 3 | 20244 | 26796 |
| ND5 | 32006 | 24 | 32734 | 64764 |
| CYTB | 21504 | 22 | 22384 | 43910 |
| gene | total_percentage | | | |
| | Uniquely mapped on chrM | Mapped to nuclear genome | common | |
| ND6 | 0.4284 | 0.0010 | 0.5706 | |
| ND1 | 0.8836 | 0.0009 | 0.1155 | |
| ND2 | 0.6071 | 0.0010 | 0.3919 | |
| COX1 | 0.1216 | 0.0005 | 0.8779 | |
| COX2 | 0.0000 | 0.0015 | 0.9985 | |
| ATP8 | 0.0000 | 0.0000 | 0.9999 | |
| ATP6 | 0.0000 | 0.0002 | 0.9998 | |
| COX3 | 0.0000 | 0.0001 | 0.9999 | |
| ND3 | 0.0000 | 0.0046 | 0.9954 | |
| ND4L | 0.0000 | 0.0001 | 0.9999 | |
| ND4 | 0.2444 | 0.0001 | 0.7555 | |
| ND5 | 0.4942 | 0.0004 | 0.5054 | |
| CYTB | 0.4897 | 0.0005 | 0.5098 | |

The total number of Ago2 CLIP-seq tags uniquely mapped on chromosome M and homologous nuclear region respectively, common tags multiply mapped on both. The percentage of each class are also shown.

**Table S3.** List of siRNAs and miRNAs used in the current study

| | Sense 5’ - 3’ | Antisense 5’ - 3’ |
|---|---|---|
| miRNA | | |
| Mmu-miR-1 | UGGAAUGUAAAGAAGUAUGUAU | ACAUACUUCUUUACAUUCAAUA |
| miR-1 5’ mutant | UCCUUACUAAAGAAGUAUGUAU | ACAUACUUCUUUAGUAAGAAUA |
| miR-1 3’ mutant | UGGAAUGUAAACUUCAUUCAAU | AGAAUGAAGUUUACAUUCAAUA |
| miR-ND1 | UAGGAAUGACCUGAAUAGUAU | ACUAUUCAGGUCAUUCCAUUA |
| miR-COX1 | UGGAAUGUAUAGGUAAUGGU | CAUUACCUAUACAUUCUUUA |
| GW182 siRNA1 | CTAATTACTCTGGCGACAAATCTT | ATTTGTCGCCAGAGTAATTAGTT |
| GW182 siRNA2 | GGATAAATCCATTCGTTAAACCTT | GTTTAACGAATGGATTTATCCTT |
| Mmu-miR-101 | UACAGUACUGUGAUAGCUGAA | CAGCUAUCACAGUACUGAUAU |
| Mmu-miR-499-5p | UUAAGACUUGCAGUGAUGUUU | ACAUCACUGCAAGUCUUCCAA |
| Mmu-miR-133b | UUUGGUCCCCUUCAACCAGCUA | GCUGGUUGAAGGGGACCAUUAU |
| Mmu-miR-450a | UUUUGCGAUGUGUUCCUAAUAU | AUUAGGAACACAUCGCAAUUAU |

**Table S4. List of PCR primers and probes**

| Primer | F 5’- 3’ | R 5’- 3’ |
|---|---|---|
| ND1 H | CCTAATGCTTACCGAACGAA | AGGGTGATGGTAGATGTGGC |
| ND1 M | TTACCAGAACTCTACTCAACT | ATCGTAACGGAAGCGTGGATA |
| ND2 M | TCAATAATTATCCTCCTGGCC | ATGATAGTAGAGTTGAGTAGC |
| ND3 M | TTCTAGTTGCATTCTGACTCC | ATAGAATTGTGACTAGAATAA |
| ND4 M | GCCTGATTACTGCCACTAATA | GGTTCCCTCATCGGGTAATAA |
| ND4L M | ACTATCACTTCTAGGGACACT | TTGGACGTAATCTGTTCCGT |
| ND5 M | AACCACACCTAGCATTCCTAC | CAGGCGTTGGTGTTGCAGGTA |
| ND6 M | ACAACTATATATTGCCGCTAC | GATATACGACTGCTATAGCTA |
| COX1 M | TCCAACTCATCCCTTGACATC | TCCTGCTATGATAGCAAACACT |
| COX2 M | CTAATTAGCTCCTTAGTCCTC | TTCGTAGCTTCAGTATCATTG |
| COX3 M | ATTCTATTCATCGTCTCGGAA | AAGGCTATGATGAGCTCATGT |
| ATP6 M | TAATCAACAACCGTCTCCATTC | GTGTCGGAAGCCTGTAATTAC |
| ATP8 M | TGCCACAACTAGATACATCAA | GGTAATGAATGAGGCAAATAG |
| CYTB M | GCAACGAAGCCTAATATTCC | TGAGATTGGTATAAGAATTAA |
| GAPDH H | GACCCCTTCATTGACCTCAAC | CTTCTCCATGGTGGTGAAGA |
| Ago2 M | AAGATCAATGTCAAACTGGGA | GTCCTGGATGATCTCCTGT |
| GAPDH M | AGGTCGGTGTGAACGGATTTG | TGTAGACCATGTAGTTGAGGTCA |
| 18S rRNA | GTAACCCGTTGAACCCCATT | CCATCCAATCGGTAGTAGCG |
| 16S rRNA | ACCGCAAGGGAAAGATGAAA | GCCACATAGACGAGTTGATTC |
| 12S rRNA | CAAACTGGGATTAGATACCCCACTAT | GAGGGTGACGGGCGGTGTGT |
| U6 | CTTCGGCAGCACATATACTA | ATATGGAACGCTTCACGAAT |
| Myogenin M | AGGTCGGTGTGAACGGATTTG | TGTAGACCATGTAGTTGAGGTCA |
| N9 | NNNNNNNNN | |
| miR-1 Probe | TACATACTTCTTTACATTCCA | |
| U6 Probe | GAATTTGCGTGTCATCCTTGCGCAGGGGCCATGCTAA | |
| Primers for reporter construction | | |
| COX1 F | ATCCTCGAGGCCCATTTCCACTATGTTCTAAACCTTGCCCATTTCCACTATGTTCTACCAAGG | |
| COX1 R | GATGCGGCCGCTAGAACATAGTGGAAATGGGCCCTTGGTAGAACATAGTGGAA | |
| ND1 F | ATTCTCGAGTACTACTATCATCAACATTCCTAACTTCTACTACTATCATCAACATTCCTCCAAT | |
| ND1 R | GAAGCGGCCGCAGGAATGTTGATGATAGTAGTATATTGGAGGAATGTTGATGA | |
| COX1 mut F | ATCCTCGAGGCCCATTTCCACTAGTAAGGAAACCTTGCCCATTTCCACTAGTAAGGACCAAG | |
| COX1 mut R | GATGCGGCCGCTCCTTACTAGTGGAAATGGGCCCTTGGTCCTTACTAGTGGAA | |
| ND1 mut F | ATTCTCGAGTACTACTATCATCAAGTAAGGTAACTTCTACTACTATCATCAAGTAAGGTCCAAT | |
| ND1 mut R | GAAGCGGCCGCACCTTACTTGATGATAGTAGTATATTGGACCTTACTTGATGATAGT | |

**Supplemental Figures and Legends**

**Figure S1** is related to main Figure 1**,** showing quantitative analysis of the mRNA levels of COX1 and ND1 during C2C12 myogenesis with 3 distinct primer sets against each transcript.

**Figure S2** is related to main Figure 2, showing the characterization and quantification of Ago2 in the mitochondria.

(A) The Trypsin protection assay performed on individual sucrose gradient fractions (3 and 4) containing mitoplasts purified from mouse heart.

(B) Results of tandem mass spectrometric analysis of Ago2 immunoprecipitated from mouse heart mitoplasts.

(C and D) Standard curves generated with baculovirus-expressed Ago2 for quantification of Ago2 in whole cell lysate and purified mitoplasts from C2C12 myoblasts and myotubes. (E) Evaluation of residual levels of non-mitochondrial mRNAs on purified mitoplasts relative to mitochondrial 16S rRNA. In comparison with the 1:1 ratio of COX1:16S rRNA and ND1:16S rRNA (thus the log10 ratio=0 in both cases), the results indicate that all non-mitochondrial mRNAs, including those encoding for mitochondrial proteins, such as NDUFB8, COX4V1, and NDUFV1, were reduced by at least 3 orders of magnitude.

(F) Quantification of Ago2 associated mtDNA-encoded transcripts by real-time RT-PCR

in purified mitoplasts. The results were presented as the percentage of each specific mRNA in Ago2 immunoprecipitant versus input from mitoplasts.

**Figure S3** is related to main Figure 3, illustrating the experimental scheme for miRACE and demonstrating the robustness of Ago2 CLIP-seq experiments on C2C12 myoblasts and myotubes.

(A) The scheme of the miRACE strategy.

(B) Identified miR-1 targets by cloning and sequencing.

(C) The reproducibility of two independent CLIP-seq experiments on C2C12 myoblasts (top panel) and myotubes (middle panel), as well as comparison between myoblasts and myotubes based on the combined datasets (lower panel).

(D) The frequency and distribution of deletions (top panel), insertions (middle panel) and substitutions (lower panel) in the Ago2 CLIP-seq tags mapped to mtDNA-encoded transcripts.

**Figure S4** is related to main Figure 4, providing a series of supporting evidence for the direct effect of miR-1 in enhancing mitochondrial translation, rather than due to indirectly induced myogenic program.

(A) Prevention of miR-1 mediated translational enhancement by a cytoplasmic sponge expressed from a Hand2-based reporter.

(B and C) miR-1 repressed the cytoplasmic reporter containing three miR-1 target sites

from ND1 and COX1. The mutant miR-1 site in the reporter lost the effect, which could be restored with the corresponding mutant miR-1 that reestablished the required base-pairing interactions.

(D and E) Transfected miR-1 alone was insufficient to induce the myogenic program on C2C12 cells.

(F) No change in mtDNA copy number in miR-1 transfected C2C12 cells.

**Figure S5** is related to main Figure 5, showing the evolutionary conservation of miR-1 target sites in ND1 and COX1 and luciferase reporter assays to characterize miR-1 target sites in the cytoplasm.

(A) Evolutionary conservation of miR-1 target sites in ND1 and COX1. Red-labeled nucleotides have base-pairing potential with miR-1.

(B and C) The designer miRND1 and miRCOX1 were effective in repressing the corresponding luciferase reporters in the cytoplasm. Note a level of partial effect of miRCOX1 on ND1 reporter because of the reduced, but not abolished base-pairing in the seed region of both COX1 and ND1 target sites in the reporters.

## Extended Experimental Procedures

### Cell culture, transfection, plasmid construction, and luciferase assay

C2C12 myoblasts were maintained in Dulbecco's minimal essential medium (DMEM; Life Technology) supplemented with 10% FBS (Life Technology). To induce C2C12 cell differentiation, cells were first grown to reach 80-90% confluency and the growth medium GM was subsequently replaced with the differentiation media DM (DMEM plus 2% horse serum). Phenotypic differentiation was typically observed after 72 to 96 hrs of culturing the cell in DM. Induced myotubes were harvested after differentiation for 4 days. HeLa cells were grown in complete DMEM plus 10% FBS.

For transfection, cells were seeded in 6-well plates and transfected using Lipofectamine 2000 (Life Technology) or TurboFect (Thermo Scientific) after cells reach 50~60% confluency. For transfection with siRNAs and miRNAs, Lipofectamine RNAiMAX (Life Technology) was used according to manufacturer's instruction. Briefly, RNA and RNAiMAX were diluted in Opti-MEM and incubated for 15 min. During this interval, cells were seeded in 24-well plates, and the transfection mix was added to the culture media. After 48 hrs, cells were harvested with Trizol for RT-PCR or SDS-loading buffer for Western.

Su9 (subunit 9 of the F0 ATPase) were amplified from a plasmid containing the gene and inserted in pcDNA3-HA-Ago2 before the HA tag. Plasmids for luciferase reporter assays were constructed by inserting target sequences into the psiCheck2 vector (Promega). The reporter was transfected into cells that had been prior treated with

control RNA or miRNAs for 12 hrs. Dual luciferase assays were performed 48 hrs after plasmid transfection using the luciferase assay kit from Promega. Standard derivation was based on triplicated experiments.

**Trypsin/Nuclease protection assays**

For Trypsin protection assays, isolated mitochondria were split into two aliquots and purified mitoplasts into three aliquots. The first aliquot was kept on ice as control; the second aliquot was treated with Trypsin (100 μg/ml), and the third aliquot of mitoplasts was treated with Trypsin (100 μg/ml) plus Triton X-100 (1%). Treated mitochondria or mitoplasts were collected by centrifugation at 18,000g for 3 min and the pellets were re-suspended in SDS-PAGE sample buffer for Western blotting.

For the nuclease protection assay, isolated mitochondria and mitoplasts were subjected to RNase T1 (3000 U/mL) plus Micrococcal nuclease (1000 U/mL) digestion on ice for 30 min in the presence or absence of Triton X-100. After the reaction, mitochondria or mitoplasts were pelleted by centrifugation, and RNA was extracted from the pellets with Trizol for RT-qPCR analysis.

**CLIP-seq and data analysis**

CLIP-seq was performed on C2C12 cells cultured in growth media or after switching to differentiation media for 4 days. The library construction was as previously described (Xue et al., 2013; Xue et al., 2009). Sequenced tags were mapped to the mouse

genome (mm10) using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). CIMS analysis was according to a published procedure (Zhang and Darnell, 2011).

**Analysis of mitochondrial translation by polysome profiling**

Sucrose gradient sedimentation of mitochondrial ribosome/polysome was carried out as described (Antonicka et al., 2013), with modifications. Briefly, mitochondria initially purified from C2C12 myoblasts or myotubes were suspended in the lysis buffer (260 mM sucrose, 100 mM KCl, 20 mM MgCl2, 10 mM Tris-pH 7.5, 1% Triton X-100, 5 mM β-mercaptoethanol, and a cocktail of protease inhibitors from Roche) on ice for 20 min. The lysate was centrifuged at 9000g for 30 min to remove particles, loaded on a 8 ml 10-30% sucrose gradient, and centrifuged at 180,000g for 260 min in a Beckman SW41-Ti rotor. To characterize the putative polysome, the lysate was pre-treated with 5 U/μL RNase I for 40 min at 25$^{o}$C. After centrifugation, 13 fractions were collected for analysis of RNA and proteins.

**Detection of nascent protein synthesis**

Click-iT reagents (Life Technology) were used to label nascent polypeptides. C2C12 myoblasts were first transfected with miR-1 or control RNA at 30-40% confluence in 24-well plates. After culturing overnight, the plate was washed 3 times with pre-warmed PBS. The cells were incubated in methionine-free DMEM for 45 min followed by the addition of 20 μM of Emetine, and incubated for another 15 min. The

media were replaced with methionine-free DMEM containing 50 μM methionine analog AHA and 20 μM Emetine. After incubation for 4 hrs, AHA-containing nascent proteins were labeled with TAMRA (Life Technology), as described (Roche et al., 2009). Labeled proteins were separated in 4-12% gradient SDS-PAGE and analyzed by Typhoon 9400. The gel was also subjected to Western blotting for VDAC and beta actin as loading controls.

**Antibodies and immunocytochemistry**

Immunoblotting, immunostaining and immunoprecipitation were performed according to standard procedures using antibodies against COX1 (Abcam, MitoSciences), ND1 (Abcam, PTGlab), beta-actin (Sigma), COX2 (Santa Cruz), COX4 (Santa Cruz), Myogenin (Santa Cruz), Ago2 (Abnova), GW182 (Santa Cruz), Lamin A/C (Santa Crutz), Tom20 (PTGlab), MHC (DSHB), HDAC4 (PTGlab), ELL2 (PTGlab), Pan-actin (Cell Signaling Technology), VDAC (Cell Signaling Technology), Tfam (PTGlab), TACO1 (PTGlab), TNRC6B (Abnova), TNRC6C (Abnova), ERp72 (PTGlab), ERp60 (PTGlab), , Histone H3 (Abcam), Tim23 (PTGlab), Hand2 (Abcam), MRPL24 (Abclonal), MRPS27 (Abclonal), HSP60 (PTGlab) and NDUFB8 (Santa Cruz).

For immunostainning, C2C12 cells were seeded in 12-well plates, fixed with 4% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and then permeabilized for 15 min on ice with 0.1% Triton X-100 in PBS. Cells were washed 3 times with PBS. Primary antibody diluted in PBS containing 3% BSA was applied to the

cell and incubated at 4°C overnight. After washing 5 times with PBS, Fluorescent-conjugated secondary antibody was applied to the coverslip and incubated in dark room for 1 hr. At this time, DAPI was applied at proper dilution, and after washing 5 times with PBS, cells were subjected to fluorescent microscopy.

**Synthetic RNA, RT-PCR, quantitative PCR, and Northern blotting**

Control and miR-1 mimics were synthesized as described (Lim et al., 2005). Specific miRNAs and siRNAs used in the study are listed in Supplemental Table S3. For PCR analysis, total RNA was extracted from cultured cells with Trizol (Life Technology). Complementary DNA was synthesized using 5μg of total RNA and the ImProme-II reverse transcription system from Promega. Gene-specific primers plus random primers (N9) were used in reverse transcription. The sequences of the forward and reverse primers for PCR are listed in Supplemental Table S4. RT-qPCR was performed with the Master SYBR Green Kit (Toyobo) on the Rotor Gene 6000 Real-time Analyzer. GAPDH and 18S rRNA served as internal controls. Northern blotting was performed as described (Chen et al., 2006).

**miRACE**

Two 150mm dishes of C2C12 cells were induced into myotubes at day 4. Cells were washed 3 times with cold PBS before harvest, scraped from the plates, and collected by centrifugation at 300g for 5 min. Collected cells were washed once with PBS and

treated with wash buffer (0.1% SDS, 0.5% deoxycholate and 0.5% NP-40 in PBS) on ice for 10 min. 500 U DNase I was added and the reaction incubated at 37$^{o}$C for 5 min. Cell lysate was clarified by centrifugation at 12,000g for 20 min at 4$^{o}$C.

The supernatant was used for IP with Ago2 antibody (Abnova) coupled on Protein G Dynal beads (Life Technology). After continuous rotation at 4$^{o}$C for 2 hrs, the supernatant was removed and beads were washed 3 times with wash buffer, twice with high-salt wash buffer (0.1% SDS, 0.5% deoxycholate and 0.5% NP-40 in 5× PBS), and finally twice with 1× PNK buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.5% NP-40). The Superscript III reverse transcriptase mix (12 μL RT buffer, 3 μL DTT, 3 μL 10 mM dNTP mix, and 3 μL Superscript III) was added to the beads, and the reaction was incubated at 37$^{o}$C for 90 min (with rotation at 1000 rpm for 15 s every 10 min on an Eppendorf thermomixer). Beads were washed twice with the PNK buffer. Poly(C) was added to nascent cDNA by using the TdT mix (44 μL DEPC $H_2O$, 12 μL tailing buffer, 3 μL 2 mM dCTP, and 1 μL TdT) and the reaction was incubated at 37 $^{o}$C for 30 min. The cDNA-miRNA chimeric molecules were isolated by phenol-chloroform extraction and amplified by PCR using a poly(G) primer and a primer complementary to miR-1. The PCR products of 100 to 200 bp in length were gel purified and cloned into the pMD-18T vector for Sanger sequencing.

**Isolation of mitochondria and purification of mitoplasts**

Cardiomyocytes from mouse heart, C2C12-derived myoblasts or myotubes were collected by centrifugation and re-suspended in ice-cold isolation buffer (250 mM

Sucrose, 10 mM Tris-HCl pH7.4, 1 mM EDTA). The cells were homogenized by 40 to 50 strokes in a pre-chilled Dounce homogenizer (Kontes). Homogenized samples were centrifuged twice at 800g for 10 min to collect post-nuclear supernatant. Mitochondria were sedimented at 13,000g for 10 min, washed once in the same buffer, and further purified by centrifugation at 40,000g for 1 hr at 4$^{o}$C on a discontinous sucrose (17%, 31%, 42%, 50%) gradient in TE buffer (10 mM Tris-HCl pH 7.4, 20 mM EDTA).

Purification of mitoplasts was according to the procedure as described (Schnaitman and Greenawalt, 1968). Briefly, stock digitonin (10 mg/ml; Sigma) solution at -20$^{o}$C was diluted to 0.5 mg/ml with isolation buffer, which was used to suspend the mitochondria pellet. After digitonin treatment on ice for 10 min, 4~5 volumes of isolation buffer were added to the reaction, and the mitochondria were pelleted by centrifugation at 18,000g at 4$^{o}$C for 3 min. The pellet was further washed 3 times with 10 volumes of isolation buffer to eliminate soluble outer membrane-associated proteins. The final pellet was designated as mitoplasts. We also further purified mitoplasts by centrifugation on a discontinuous sucrose gradient. In this procedure, digitonin-treated mitochondria were re-suspended in isolation buffer and then layered onto a discontinuous sucrose gradient consisting of 25.2%, 37.7%, 51.1%, and 61.5% layers followed by centrifugation at 77,000g for 90 min at 4$^{o}$C. Western blotting analysis of mitochondrial outer and inner membrane proteins indicate that direct sedimentation and purification through the sucrose gradient produced identical results.

**Trypsin/Nuclease protection assays**

For Trypsin protection assays, isolated mitochondria were split into two aliquots and purified mitoplasts into three aliquots. The first aliquot was kept on ice as control; the second aliquot was treated with Trypsin (100 μg/ml), and the third aliquot of mitoplasts was treated with Trypsin (100 μg/ml) plus Triton X-100 (1%). Treated mitochondria or mitoplasts were collected by centrifugation at 18,000g for 3 min and the pellets were re-suspended in SDS-PAGE sample buffer for Western blotting.

For ribo-IP experiments, purified mitoplasts from mouse heart was lysed in wash buffer (0.1% SDS, 0.5% deoxycholate and 0.5% NP-40 in PBS) on ice for 15 min followed by treatment with DNase I (Promega) at the final concentration of 500 U/200μL at 37°C for 10 min. The supernatant was clarified by centrifugation at 12,000g for 20 min and 200 μL of mitochondrial lysate was added to 50μL Dynal beads (Life Technology) either coupled with 2μg anti-Ago2 antibody or control IgG. After rotation at $4^{o}$C for 2 hrs, the beads were pelleted; the supernatant removed, and the beads were washed 6 times with wash buffer and twice with high-salt wash buffer (0.1% SDS, 0.5% deoxycholate and 0.5% NP-40 in 5 × PBS). RNA was extracted using Trizol (Life Technology) and analyzed by real-time RT-PCR.

For the nuclease protection assay, isolated mitochondria and mitoplasts were subjected to RNase T1 (3000 U/mL) plus Micrococcal nuclease (1000 U/mL) digestion on ice for 30 min in the presence or absence of Triton X-100. After the reaction, mitochondria or mitoplasts were pelleted by centrifugation, and RNA was extracted from

the pellets with Trizol for RT-qPCR analysis.

**Quantification of Ago2, miR-1 and mtDNA**

The relative mtDNA copy number was calculated as a ratio of mtDNA/nuclear (n) DNA according to a previous study (Duchene et al., 2009). Cells were lysed in the RIPA buffer and DNA extracted with phenol/chloroform followed by ethanol precipitation. For quantification of mtDNA, we used a pair of primers (mtDNA forward, 5'-CCTATCACCCTTGCCATCAT-3' and mtDNA reverse, 5'-GAGGCTGTTGCTTGTGTGAC-3') that target the COX1 gene for qPCR analysis. To quantify nDNA, we used a pair of primers (nDNA forward, 5'-ATGGAAAGCCTGCCATCATG-3' and nDNA reverse, 5'-TCCTTGTTGTTCAGCATCAC-3') that target the nuclear Pecam gene for qPCR analysis.

His-tagged recombinant Ago2 protein (>95% purity; Sino Biological) was used to generate a standard curve by Western blotting. Whole cell lysate from C2C12 myoblasts or myotubes and lysate of Trypsin-treated mitoplasts were similarly analyzed. The copy number of Ago2 was determined by using the TotalLab quantification program. To quantify the copy number of miR-1 in C2C12 cells before and after differentiation or in purified mitoplasts, we generated a standard curve by RT-qPCR using serially diluted synthetic miR-1 (Ribo Bio) and then performed quantitative analysis of miR-1 in whole cell lysate or purified mitoplasts.

To determine the copy number of Ago2 or miR-1 per nucleus or per mitochondrial genome, we generated a standard curve by qPCR against a 600 bp nuclear DNA (Pecam) and 1,544 bp (full length) COX1 DNA, respectively. We used these standard curves to calculate the copy number of Ago2 and miR-1 based on Western blotting or RT-qPCR analysis performed on the same batch of Trypsin-treated mitoplasts and total cell lysate.

**Measurement of mitochondrial activities**

The ATP assay kit was from Beyotime and the assay was performed according to manufacturer's instruction. After centrifugation to remove cell debris, the supernatant was added to the substrate solution. The luminescence was recorded in an Illuminometer with an integration time of 10 sec per well.

The activity of NADH-ubiquinone oxidoreductase (Complex I) was assayed as described (Frost et al., 2005). Briefly, isolated mitochondria were frozen and thawed to cause gentle opening. The mix was added to a 10 mM potassium phosphate buffer (pH 8.0) containing 1 μM antimycin A and 0.2 mM NADH). The rate of NADH oxidation was followed at 340 nm in a UV spectrophotometer (Bio-Rad). After absorbance was recorded for 1 min, ubiquinone was added at the final concentration of 70 μM, and the stimulated rate of NADH oxidation (Complex I activity) was followed for another 2 min. The Complex I activity was calculated from the slope of absorbance decrease over time using an extinction coefficient for NADH. The cytochrome c oxidase activity (Complex

IV) was assayed by measuring oxidation of cytochrome c at 550 nm using a cytochrome c oxidase activity kit (Genmed).

**Determination of mitochondrial protein stability**

To determine the stability of mitochondrial proteins, the mitochondrial translation inhibitor Chloramphenicol INN (50 μg/mL) was included in the culture media for control RNA or miR-1 transfected C2C12 cells. Whole cell lysate was obtained at different time points (0 min, 2 hrs, 4 hrs, 8 hrs, 16 hrs, 32 hrs), fractionated by 12% SDS-PAGE and subjected to Western blotting for ND1 and Actin. The Western blotting signals were quantified by using the program from TotalLab and the levels of ND1 were compared against that at the 0 min treatment point.

**References**

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