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**Age-related decline in murine heart and skeletal muscle performance is attenuated by reduced Ahnak1 expression**

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**Supporting information: Materials and Methods**

***In-vitro* investigations using a human adult left ventricular cardiomyocyte cell line, the AC16 cells**

AC16 cells were cultured in DMEM/F12 (InvitrogenTM), supplemented with 12.5% FBS (PAA Laboratories), penicillin/streptomycin (100 U/mL, 100 U/mL, PAA) and Amphotericin B (0.25 µg/mL, Invitrogen) at 37°C in 5% CO2 before functional analysis. For transient knock-down of Ahnak1, a set of three different Stealth siRNAs (small interfering RNA) against human Ahnak1 (HSS149070, HSS149071, HSS149072, ThermoFisher Scientific) were transfected into AC16 cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The Stealth siRNA negative control (12935112, ThermoFisher Scientific) was used to evaluate the siRNA specificity (scramble-siRNA). Mock-transfected cells (treated with transfection reagent only) were employed to verify any non-specific effects caused by the transfection reagent or process. Briefly, siRNA (10µM each) and Lipofectamine RNAiMAX were separately diluted in Opti-MEM (Invitrogen Life Technologies) and then combined and gently mixed, followed by 5min incubation at room temperature (RT). The 35μl siRNA-lipofectamine complex was added to 8x103 cells (for Seahorse experiments) or 6x103 cells (for gene/protein expression) in each well (96-well plate) with 200µl DMEM/F12 and incubated at 37°C with 5% CO2 for 48h or 72h, respectively. After transfection, cells were analyzed for mRNA and protein expression and mitochondrial respiration.

**Oxygen consumption rate**

Freshly isolated cardiomyocytes from aged adult female and male Ahnak1-KO and WT hearts were seeded in XF96 cell culture microplates (5x103/well). After 2h, when cells were attached, unbuffered DMEM XF medium supplemented with 2mM glutamine, 2mM sodium pyruvate and 10mM glucose (pH 7.4) was added to the cells and placed in a 37°C CO2-free incubator for 30min. After OCR baseline measurements, oligomycin (0.5µM), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone, 1.0μM), and rotenone plus antimycinA (4.0μM each) were sequentially added into each well and OCRs were measured. Twelve replicates were run for each genotype and sex per heart. The measurements were performed with 7 murine hearts/genotype/sex.

AC16 cells transfected with Ahnak1-siRNA, neg-siRNA or mock-transfected: Approximately 8x103 AC16 cells/well were seeded in XF96 cell culture microplates. After 48h, supplemented unbuffered DMEM XF medium was added to the cells and placed in a 37°C CO2-free incubator for 1h. After baseline measurements of OCR, oligomycin (1.0μM), FCCP (1.0μM), and rotenone plus antimycinA (2.0μM each) were added sequentially to each well and OCRs were measured. Twelve replicates were run for each condition, and experiments were repeated 7 times independently.

Upon completion of the assays, data were normalized for protein content of cells. OCR profiles were expressed as pmol O2/min/µg protein. All parameters (the rates of basal respiration, max. respiration, spare (reserve) respiratory capacity (the difference between the max. respiration and the basal mitochondrial respiration), and ATP production) were calculated using Wave Software according to manufacturer (Agilent Technologies).

**Mitochondrial isolation for electron microscopy and for mitochondrial sub-fractionation analysis**

Mitochondria and fractions of mitochondrial matrix and mitochondrial membrane were isolated from freshly excised hearts of aged adult Ahnak1-KO and WT littermates of both sexes (n=3-4) with Mitochondria Isolation Kit for Tissue (Thermo Scientific™) according to manufacturer’s instruction. Heart tissue was homogenized in 800μl of Reagent A solution on ice using a glas/glas Dounce homogenizer (30 strokes). After adding 800μl of Mitochondria Isolation Reagent C, collected homogenate was spun at 700xg for 10min at 4°C. Supernatant was further spun at 3.000xg for 15min at 4°C. Mitochondrial pellet was washed with 500µl wash buffer and spun at 12.000xg for 5min. The final pellet (mitochondrion-enriched fraction) was further processed for either electron microscopy analysis or for separating mitochondrial matrix and membrane fractions. To separate mitochondrial membrane and matrix fractions, the mitochondrial pellet was treated with 300-1000µl of 2% (w/v) CHAPS/TBS, vortexed for 1min, and spun at high speed for 2min. The supernatant contains soluble mitochondrial protein (matrix) whose protein concentration was analyzed by Pierce BCA Protein Assay. The pellet contains insoluble mitochondrial membrane fractions. Subsequently, both mitochondrial matrix and membrane fractions were resuspended in RIPA buffer and prepared for Western blot analysis. The purity of fractions was assessed by Western blot analysis using antibodies against mitochondrial TIM23 (translocase of the inner membrane, 611222, BD Bioscience) and cytosolic tubulin (T9026, Sigma) marker proteins.