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Supplemental information

Exogenous expression of ATP8, a mitochondrial encoded protein, from the nucleus *in vivo*

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Figure S1

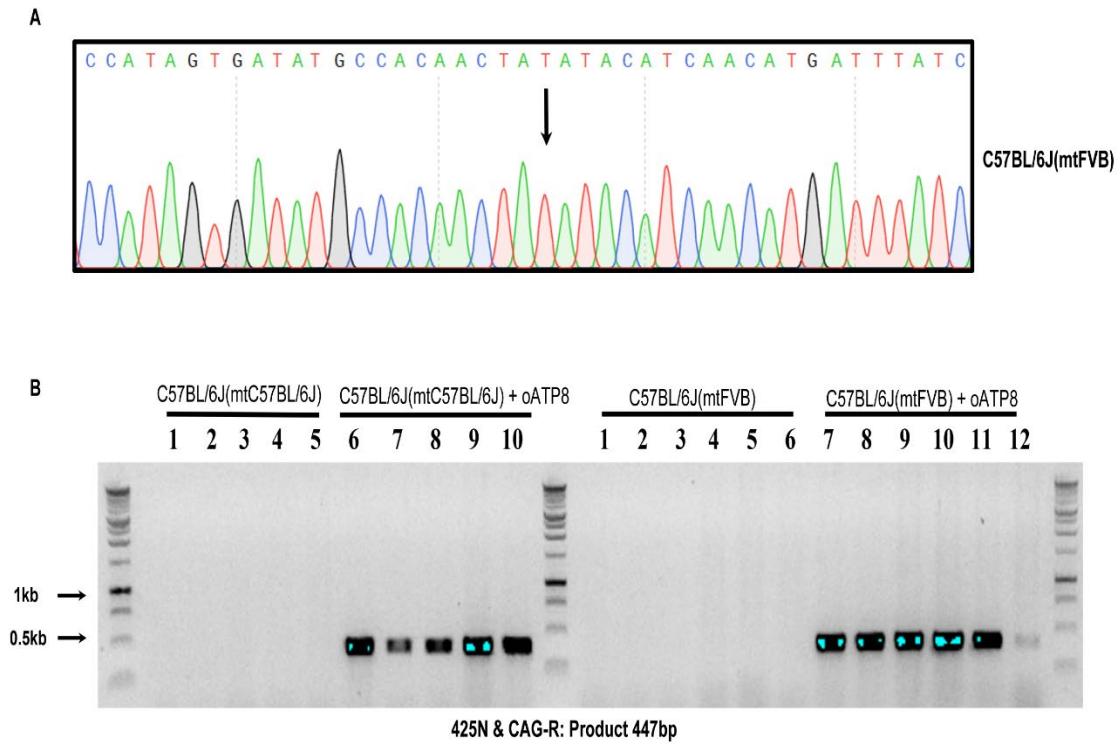
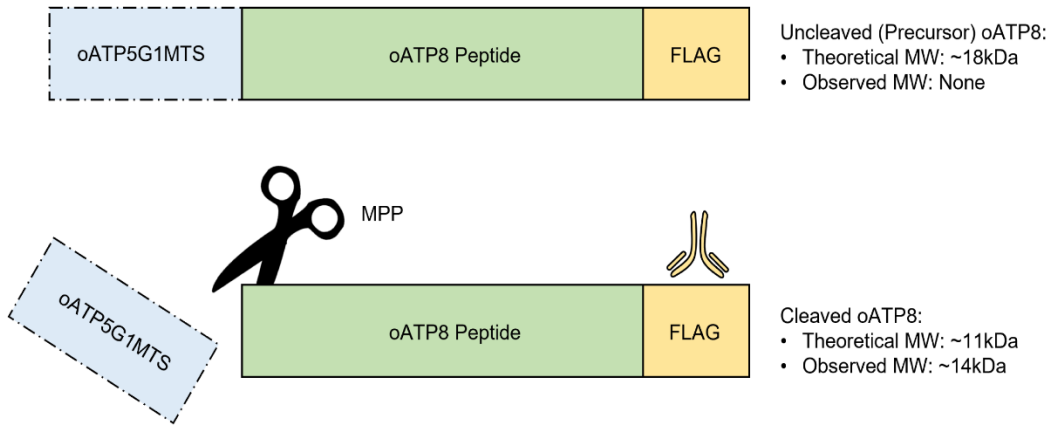


Figure S1: Chromatogram showcasing Sanger sequencing verification of the m.7778G-T polymorphism present in the mitochondrial genome of C57BL/6J(mtFVB) animals (S1A). Additional PCR results confirming the presence of the oATP8 construct in the nuclear genome (S1B).

Figure S2

A



B

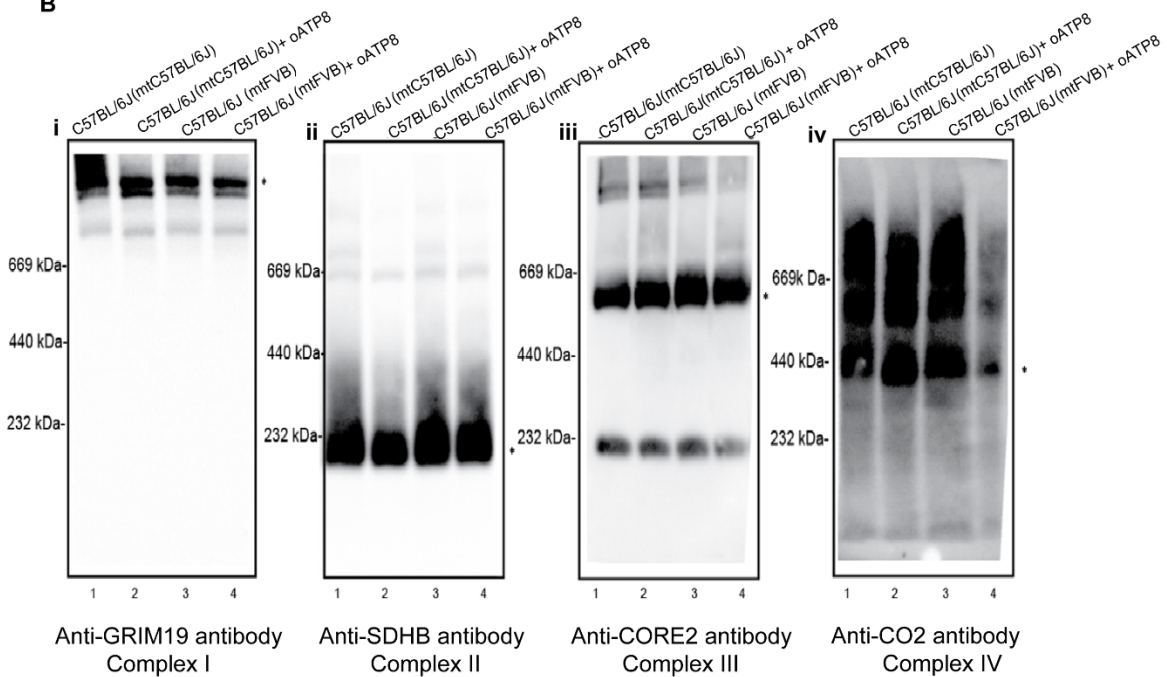


Figure S2: Schematic representation of the theoretical and observed molecular weights of the oATP8 protein when the ATP5G1 MTS is not fully processed (top) and when the MTS is cleaved (bottom) upon translocation into the mitochondria (S2A). Blue-Native PAGE western blots using 25 μ g protein from mouse liver mitochondrial fractions of 12-week old non-transgenic and transgenic C57BL/6J(mtC57BL/6J) and C57BL/6J(mtFVB) strains (S2B) (n = 3).

Figure S3

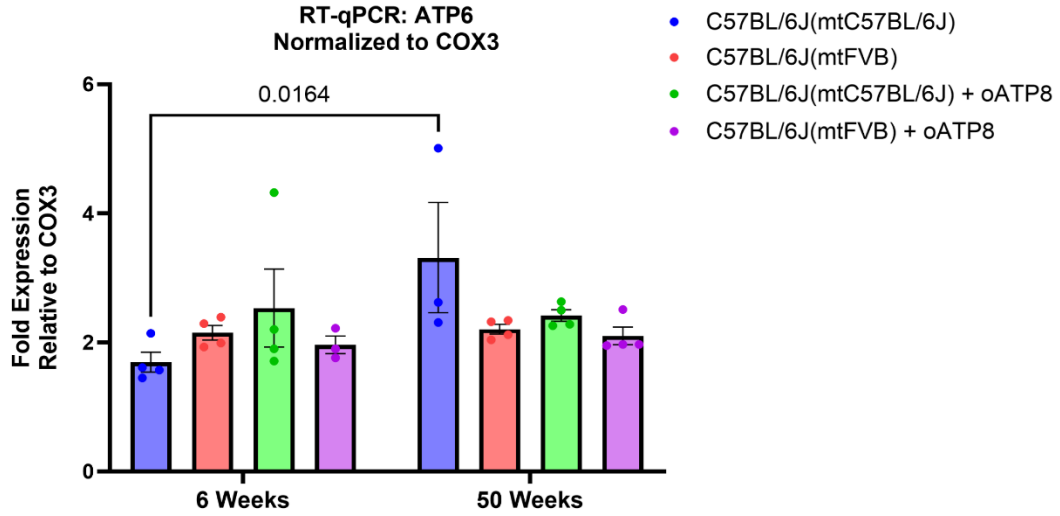


Figure S3: Quantitative RT-PCR detection of mRNA levels for endogenous ATP6 from brain from 50-week old mice. Endogenous ATP6 mRNA was normalized to COX3, a mitochondria-encoded gene (S3) (n = 3-4 animals, performed in triplicate). Error bars show SEM. Two-way ANOVA was performed; P = 0.0165.

Figure S4

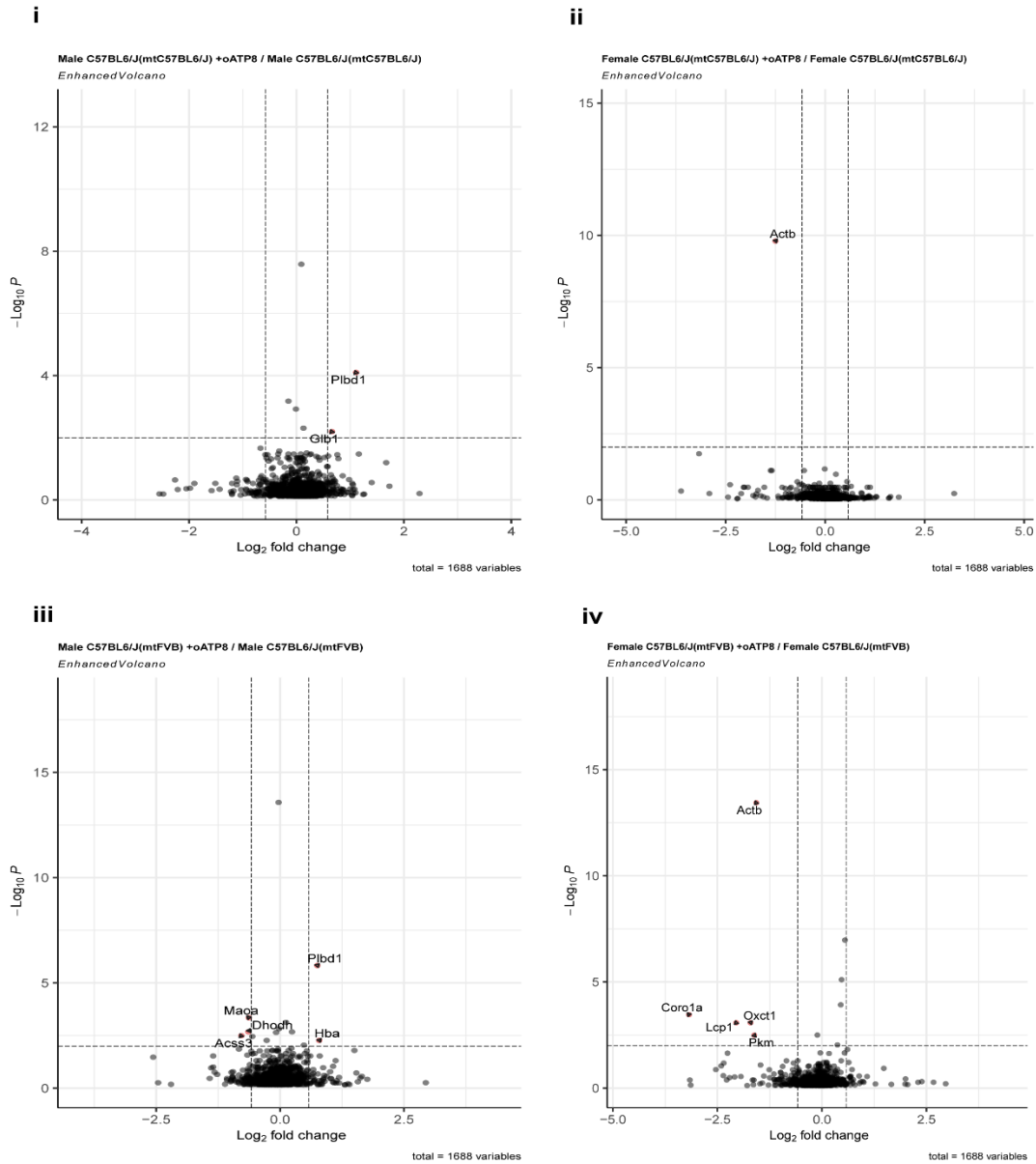


Figure S4: Volcano plot representing global proteomic profiles of 12-week old mice with the following comparisons: (S4i) Male Control Transgenic / Male Control Non-Transgenic; (S4ii) Female Control Transgenic / Female Control Non-Transgenic; (S4iii) Male Mutant Transgenic / Male Mutant Non-Transgenic; (S4iv) Female Mutant Transgenic / Female Mutant Non-Transgenic (n = 3 animals). A total of 1688 proteins were detected. Significantly altered proteins were determined using an absolute average \log_2 ratio > 0.58 and Q value < 0.01. Labels represent genes.

Table S1: ATP Hydrolysis Activity

Strain	Vmax	95% CI ^a	Km	95% CI ^a
C57BL/6J(mtC57BL/6J)	189.5	168.3 – 213.9	2.799	1.583 – 4.563
C57BL/6J(mtFVB)	201.8	184.4 – 221.1	3.426	2.369 – 4.799
C57BL/6J(mtC57BL/6J) + oATP8	205	192.7 – 218.2	2.857	2.123 – 3.762
C57BL/6J(mtFVB) + oATP8	216.6	196.6 – 239.0	3.319	2.197 – 4.817

^a 95% confidence interval

Table S2: Oligomycin Sensitivity

Strain	IC50	95% CI ^a
C57BL/6J(mtC57BL/6J)	0.4881	0.4208 – 0.5472
C57BL/6J(mtFVB)	0.4641	0.3922 – 0.5325
C57BL/6J(mtC57BL/6J) + oATP8	0.5125	0.4833 – 0.5421
C57BL/6J(mtFVB) + oATP8	0.4287	0.3767 – 0.4718

^a 95% confidence interval

Table S3: Open Field Test

Metric	C57BL/6J(mtC57BL/6J)	C57BL/6J(mtC57BL/6J) + oATP8	C57BL/6J(mtFVB)	C57BL/6J(mtFVB) + oATP8
Total Moves	386 + 14.4	383 + 21.8	387 + 20.5	397 + 26.6
Total Distance (cm)	865 + 204	862 + 170	874 + 191	864 + 156
Total Distance in Margin (cm)	293 + 87.5	312 + 80.6	324 + 108	320 + 74.5
Total Distance in Center (cm)	209 + 88.0	182 + 70.8	181 + 58.9	190 + 68.1

Parameters assessed using open field. C57BL/6J(mtC57BL/6J) (n=20), C57BL/6J(mtC57BL/6J) + oATP8 (n=16), C57BL/6J(mtFVB), (n=20), C57BL/6J(mtFVB)+ oATP8 (n=18). Mean + SD is described.

Table S4: Elevated Plus Maze Test

Metric	C57BL/6J(mtC57BL/6J)	C57BL/6J(mtC57BL/6J) + oATP8	C57BL/6J(mtFVB)	C57BL/6J(mtFVB) + oATP8
Total Distance (cm)	2780 + 676	2970 + 682	2620 + 599	2400 + 518
Entries into Closed Arm	40.6 + 12.0	51.2 + 15.0	40.9 + 11.4	37.0 + 11.6
Time in Closed Arm (seconds)	514 + 45.4	497 + 49.2	503 + 45.8	528 + 31.0
Percent of Total Time in Closed Arm	85.6 + 7.57	82.9 + 8.21	83.9 + 7.64	87.9 + 5.16
Entries into Open Arm	15.2 + 7.72	21.1 + 16.1	14.4 + 8.54	11.9 + 6.04
Time in Open Arm (seconds)	34.1 + 26.9	39.5 + 26.0	33.3 + 24.5	27.2 + 15.8
Percent of Total Time in Open Arm	5.68 + 4.49	6.58 + 4.33	5.55 + 4.09	4.53 + 2.64

Parameters assessed using elevated plus maze. C57BL/6J(mtC57BL/6J) (n=16), C57BL/6J(mtC57BL/6J) + oATP8 (n=15), C57BL/6J(mtFVB), (n=17), C57BL/6J(mtFVB) + oATP8 (n=18). Mean + SD is described.

Table S5: Metabolic Cage – Full Day Cycle

Metric	C57BL/6J(mtC57BL/6J)	C57BL/6J(mtC57BL/6J) + oATP8	C57BL/6J (mtFVB)	C57BL/6J(mtFVB)+ oATP8
Total Distance (cm)	272 + 52.5	312 + 104	237 + 55.8	287 + 239
Non-Wheel Activity	206 + 45.9	250 + 85.4	190 + 44.6	224 + 200
Wheel Counts	7.97 + 4.34	7.20 + 6.34	9.69 + 6.52	7.31 + 4.46
Energy Balance	17.7 + 5.27	21.9 + 3.87	19.9 + 3.35	17.3 + 8.82
Energy Expenditure	0.381 + 0.0376	0.397 + 0.0285	0.362 + 0.0535	0.396 + 0.0198

Oxygen Consumption	77.0 + 7.59	80.5 + 5.71	73.2 + 10.8	80.4 + 4.10
Carbon Dioxide Production	69.6 + 7.01	72.5 + 5.52	66.2 + 9.82	72.0 + 3.57
Respiratory Exchange Ratio	0.894 + 0.0132	0.893 + 0.0161	0.898 + 0.0183	0.888 + 0.0181
Hourly Food Intake	18.1 + 5.24	22.3 + 3.88	20.3 + 3.35	17.7 + 8.81
Hourly Water Intake	6.25 + 1.11	6.02 + 1.12	5.25 + 1.30	5.95 + 0.987

Parameters assessed using metabolic cage (n=8 animals). Mean + SD is described.

Table S6: Metabolic Cage – Light Cycle

Metric	C57BL/6J(mtC57 BL/6J)	C57BL/6J(mtC57 BL/6J) + oATP8	C57BL/6J(mt FVB)	C57BL/6J(mtF VB) + oATP8
Total Distance (cm)	258 + 51.1	296 + 97.6	225 + 52.9	271 + 227
Non-Wheel Activity	197 + 45.6	238 + 80.2	180 + 42.3	212 + 190
Wheel Counts	2.49 + 1.98	2.13 + 1.72	2.52 + 1.98	1.45 + 1.22
Energy Balance	4.68 + 1.93	6.25 + 1.97	5.52 + 0.941	6.17 + 3.85
Energy Expenditure	0.347 + 0.0394	0.361 + 0.0209	0.327 + 0.0448	0.363 + 0.0200
Oxygen Consumption	70.9 + 8.06	73.6 + 4.23	66.7 + 9.14	74.4 + 4.26
Carbon Dioxide Production	61.3 + 6.90	63.8 + 3.94	57.8 + 8.04	63.6 + 2.98
Respiratory Exchange Ratio	0.859 + 0.00945	0.862 + 0.0156	0.862 + 0.0215	0.851 + 0.0158
Hourly Food Intake	5.02 + 1.91	6.61 + 1.98	5.85 + 0.937	6.53 + 3.85
Hourly Water Intake	1.96 + 0.599	1.84 + 0.657	1.39 + 0.621	1.70 + 0.522

Parameters assessed using metabolic cage only during the light cycle (n=8 animals). Mean + SD is described.

Table S7: Metabolic Cage – Dark Cycle

Metric	C57BL/6J(mtC57 BL/6J)	C57BL/6J(mtC57 BL/6J) + oATP8	C57BL/6J(mt FVB)	C57BL/6J(mtF VB) + oATP8
Total Distance (cm)	13.0 + 2.77	14.2 + 4.43	12.1 + 3.31	14.3 + 11.5
Non-Wheel Activity	9.87 + 2.38	11.4 + 3.68	9.67 + 2.66	11.1 + 9.62
Wheel Counts	0.622 + 0.393	0.554 + 0.547	0.863 + 0.705	0.645 + 0.423
Energy Balance	0.572 + 0.181	0.664 + 0.147	0.678 + 0.185	0.518 + 0.311
Energy Expenditure	0.0189 + 0.00251	0.0190 + 0.00235	0.0192 + 0.00423	0.0209 + 0.000968
Oxygen Consumption	3.79 + 0.501	3.82 + 0.473	3.86 + 0.852	4.20 + 0.202
Carbon Dioxide Production	3.56 + 0.490	3.56 + 0.447	3.62 + 0.785	3.92 + 0.175
Respiratory Exchange Ratio	0.932 + 0.0200	0.927 + 0.0201	0.936 + 0.0181	0.928 + 0.0242
Hourly Food Intake	0.591 + 0.180	0.683 + 0.148	0.697 + 0.188	0.539 + 0.311
Hourly Water Intake	0.194 + 0.0388	0.180 + 0.0361	0.184 + 0.0320	0.206 + 0.0418

Parameters assessed using metabolic cage only during the dark cycle (n=8 animals). Mean + SD is described.

Table S8: Treadmill Exhaustion Assay:

Metric	C57BL/6J(mtC57BL/6J)	C57BL/6J(mtC57BL/6J) + oATP8	C57BL/6J(mtFVB)	C57BL/6J(mtFVB) + oATP8
Total Time (seconds/g)	67.0 + 18.3	50.5 + 14.7	69.9 + 8.73	58.5 + 11.4
Distance (cm/g)	22.6 + 7.73	15.5 + 7.12	24.6 + 3.43	18.2 + 6.25

Parameters assessed using treadmill exhaustion assay. Each of the values are normalized to body weight. C57BL/6J(mtC57BL/6J) (n=7), C57BL/6J(mtC57BL/6J) + oATP8 (n=14), C57BL/6J(mtFVB), (n=8), C57BL/6J(mtFVB) + oATP8 (n=13). Mean + SD is described.

Table S9: Fertility of Transgenic Animals is not Affected:

Parent (Male)	Parent (Female)	Average No. Offspring (Total)	SEM	Average No. Offspring (Male)	SEM	Average No. Offspring (Female)	SEM
C57BL/6J(mtC57BL/6J)	C57BL/6J(mtFVB)	6.38	0.47	3.44	0.33	3.12	0.29
C57BL/6J(mtC57BL/6J) + oATP8	C57BL/6J(mtC57BL/6J)	6.70	0.40	2.89	0.33	4.00	0.57
C57BL/6J(mtC57BL/6J)	C57BL/6J(mtC57BL/6J) + oATP8	5.40	0.71	3.20	0.28	3.14	0.37
C57BL/6J(mtC57BL/6J) + oATP8	C57BL/6J(mtFVB)	7.71	0.44	3.88	0.37	3.50	0.50

Table S10: Genotyping PCR primers

Primer Name	Sequence	PCR Product Size
CAG - Forward PolyA - Reverse	5'GCCTCTGCTAACCATGTTTCATGCCTTCTTC3' 5'ATGTGGTATGGCTGATTATGATCAGTTATCTAG 3'	714 bp
425N - Forward CAG - Reverse	5'GGTGATAGGTGGCAAGTGGTATTCCGTAAG3' 5'CATATATGGGCTATGAACTAATGACCCCGT3'	447 bp
ROSA26 - Forward* CAG - Reverse	ASC Proprietary Sequence (AST-2006-MT150-a) 5'CATATATGGGCTATGAACTAATGACCCCGT3'	635 bp
PolyA - Forward ROSA26 - Reverse*	5'CTAGATAACTGATCATAATCAGCCATACCACAT 3' ASC Proprietary Sequence (AST-2006-MT150-d)	687 bp

* Proprietary primers acquired from ASC.

Table S11: Quantitative RT-PCR primers

Primer Name	Sequence
COX3-Forward COX3-Reverse	5'ACACATGATCTAGGAGGCTGC3' 5'AAGCTTGGAGGATGGTGAAGT3'
COX10-Forward COX10-Reverse	5'GAAGAAAGGCTTTGGCCGTG3' 5'GGCCGCAACAAGTCCTAGAT3'
Endo-ATP8-Forward* Endo-ATP8-Reverse*	5'TGCCACAACCTAGATACATCAACA3' 5'AGGTGCCAGTGGGAATGTTT3'
Exo-ATP8-Forward* Exo-ATP8-Reverse*	5'GCTGGACACCTCTACATGGTT3' 5'TCACCTTCATTGTGGTCAGGG3'
ATP6-Forward ATP6-Reverse	5'GCAGTCCGGCTTACAGCTAA3' 5'GGTAGCTGTTGGTGGGCTAA3'

*Endo-ATP8= mitochondrially encoded ATP8; Exo-ATP8= exogenous allotopic ATP8

Table S12: Mass Spectrometry of all samples using direct-DIA quantification.**Table S13: Mass Spectrometry Protein Quantification data**

Table S14: Mass Spectrometry Peptide Quantification data**Table S15: Statistical analysis**

Figures/Tables	Tests
Fig 3A: ATP8 expression in different tissues, C57BL/6J (mt C57BL/6J) and C57BL/6J (mt C57BL/6J) + oATP8	Values are expressed as means \pm standard error of the mean
Fig 3B: oATP8 expression in different tissues, C57BL/6J (mt FVB) and C57BL/6J(mt FVB)+ oATP8	Values are expressed as means \pm standard error of the mean
Fig 4A: ATP8 expression in mitochondrial fractions	Values are expressed as means \pm standard error of the mean
Fig 5A: Quantitative RT-PCR detection of mRNA levels for transgenic oATP8 normalized to Actin	Error bars show SEM. Two-way ANOVA was performed
Fig 5B: Quantitative RT-PCR detection of mRNA levels for transgenic oATP8 normalized to Cox10	Error bars show SEM. Two-way ANOVA was performed
Fig 5C: Quantitative RT-PCR detection of mRNA levels for endogenous oATP8 normalized to Cox3	Error bars show SEM. Two-way ANOVA was performed
Fig 6B oATP8 and endogenous ATP8 expression in mouse liver tissue from 6-, 12-, 30-, and 50-week-old transgenic C57BL/6J(mtC57BL/6J) and C57BL/6J(mtFVB) mice	Error bars show SEM. Two-way ANOVA with Šídák's multiple comparisons. $P > 0.05$: NS (not significant); $P = 0.0018$: **; $P \leq 0.0001$: ****.
Fig 6C Apparent oATP8/ATP8 ratio in mouse liver tissue from 6-, 12-, 30-, and 50-week-old transgenic C57BL/6J(mtC57BL/6J) and C57BL/6J(mtFVB) mice	Error bars show SEM. Two-way ANOVA with Šídák's multiple comparisons. $P > 0.05$: NS (not significant); $P = 0.0205$: *; $P = 0.00015$: **.
Fig 6E: oATP8 and endogenous ATP8 expression in mouse liver mitochondria from transgenic C57BL/6J(mtC57BL/6J) and C57BL/6J(mtFVB) mice	Error bars show SEM. Two-way ANOVA with Šídák's multiple comparisons. $P > 0.05$: NS (not significant); $P = 0.0114$: *.
Fig 6F: Apparent oATP8/ATP8 ratio in mouse liver mitochondria from transgenic C57BL/6J(mtC57BL/6J) and C57BL/6J(mtFVB) mice	Error bars show SEM. Unpaired t test with Welch's correction. $P > 0.05$: NS (not significant).

Fig 7B Quantitative assessment of ATP hydrolysis activity from liver mitochondria from 12-week-old male mice	Vmax and Km values were calculated with 95% CI
Fig 7C Oligomycin sensitivity	IC50 values were calculated with 95% CI
Fig 7D: ATP synthesis and maximal respiratory capacity were monitored in isolated mitochondria from 9 - 12-week-old male mice	Error bars show SEM. Two -way ANOVA was performed
Fig 7E: ATP synthesis and maximal respiratory capacity were monitored in skeletal muscle mitochondria from 9 - 12-week-old male mice	Error bars show SEM. Two -way ANOVA was performed
Figure S3 Quantitative RT-PCR detection of mRNA levels for endogenous ATP6 from brain from 50-week old mice	Error bars show SEM. Two-way ANOVA was performed; P = 0.0165.
Table S1 ATP Hydrolysis Activity	Vmax and Km values were calculated with 95% CI
Table S2 Oligomycin sensitivity	IC50 values were calculated with 95% CI
Table S3 Open Field Test	Values are expressed as means \pm SD
Table S4 Elevated Plus Maze Test	Values are expressed as means \pm SD
Table S5 Metabolic Cage – Full Day Cycle	Values are expressed as means \pm SD
Table S6 Metabolic Cage – Light Cycle	Values are expressed as means \pm SD
Table S7 Metabolic Cage – Dark Cycle	Values are expressed as means \pm SD
Table S8 Treadmill Exhaustion Assay	Values are expressed as means \pm SD
Table S9 Fertility of Transgenic Animals is not Affected	Values are expressed as means \pm SD
Table S12 Mass Spectrometry of all samples using direct-DIA quantification	a paired t-test, and p-values were corrected for multiple testing, using the Storey method
Table S13 Mass Spectrometry Protein Quantification data	a paired t-test, and p-values were corrected for multiple testing, using the Storey method
Table S14 Mass Spectrometry Peptide Quantification data	a paired t-test, and p-values were corrected for multiple testing, using the Storey method