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Supplemental Information

Mitochondrial Diagnostics: A Multiplexed Assay Platform for Comprehensive Assessment of Mitochondrial Energy Fluxes

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Supplemental Figure 1.

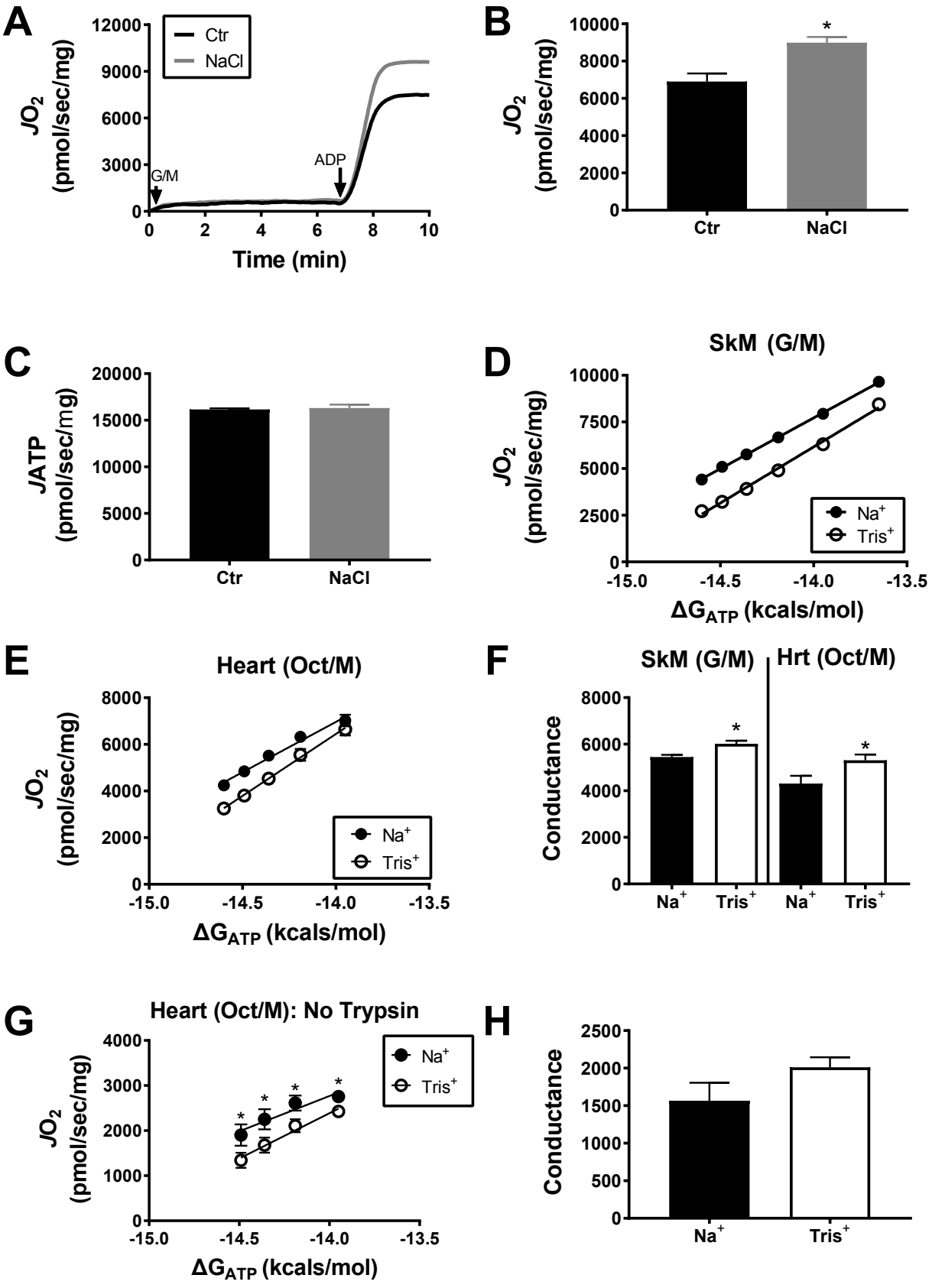
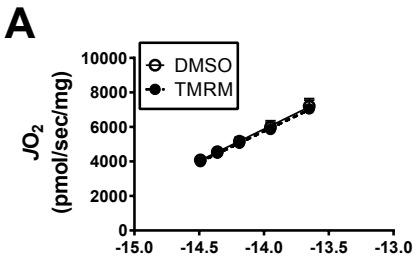
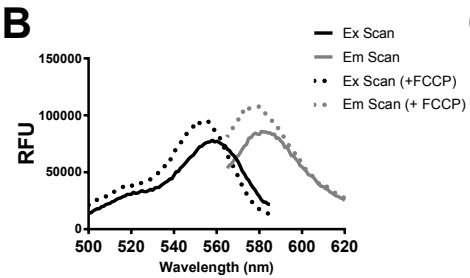


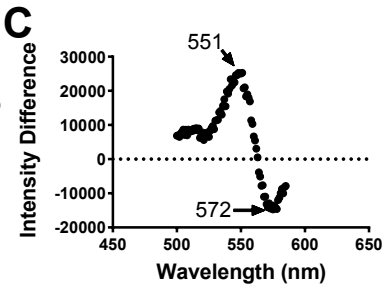
Figure S1. The presence of Na^+ impairs respiratory conductance. Related to Figure 1 and Star Methods – "Mitochondrial Respiratory Control".

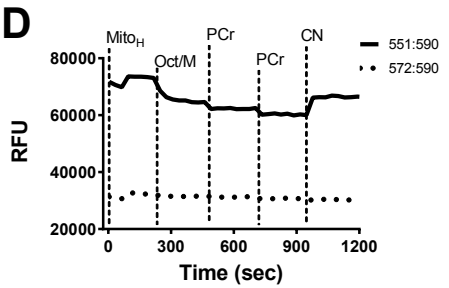
(**A**) Representative trace displaying oxygen consumption in skeletal muscle mitochondria pre-incubated with NaCl (10 mM) or vehicle and energized with G/M (10/2.5 mM), followed by the addition of ADP (1 mM). (**B**) ADP-dependent JO_2 in skeletal muscle mitochondria respiring on G/M and pre-incubated with NaCl or vehicle. (**C**) JATP synthesis in skeletal muscle mitochondria energized with G/M in the absence or presence of NaCl (10 mM). (**D-E**) Phosphocreatine titration experiments performed in skeletal muscle mitochondria energized with G/M (**D**) and heart mitochondria energized with Oct/M (**E**) in which the PCr was supplied as either di-sodium phosphocreatine (Na^+) or di-tris phosphocreatine ($Tris^+$). ATP in each experiment was added as either a sodium or tris salt for consistency. (**F**) Calculated linear slopes (i.e., respiratory conductance) from the experiments in panels F-G. (**G-H**) Phosphocreatine titration experiments performed in heart mitochondria prepared without trypsin or EDTA and energized with Oct/M in which the PCr was supplied as either di-sodium phosphocreatine (Na^+) or di-tris phosphocreatine ($Tris^+$). ATP in each experiment was added as either a sodium or tris salt for consistency. (**H**) Calculated linear slopes (i.e., respiratory conductance) from the experiments in panels G. Data are mean ± SEM. Differences between groups were analyzed by unpaired 2-tailed t-tests. *P<0.05. N=3-5/group, where "N" represents 3-4 biological replicates (i.e., mitochondrial preparations).

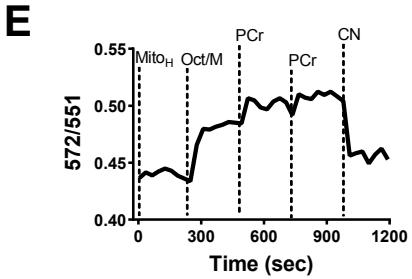
Supplemental Figure 2.

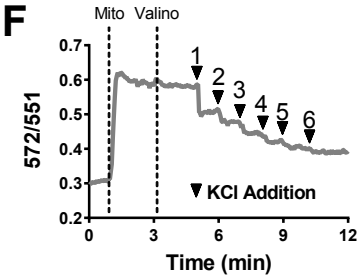




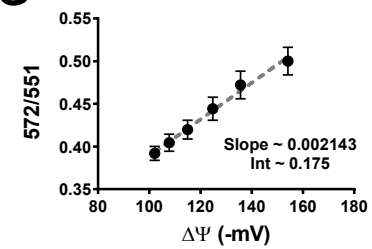


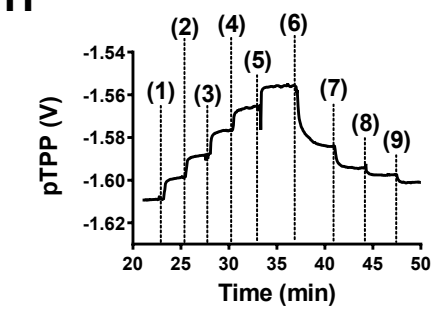




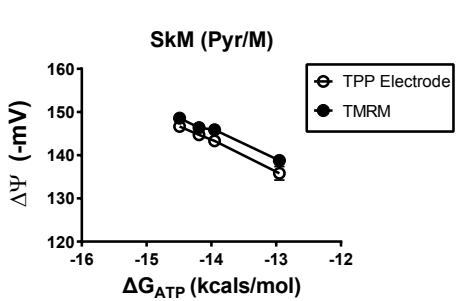


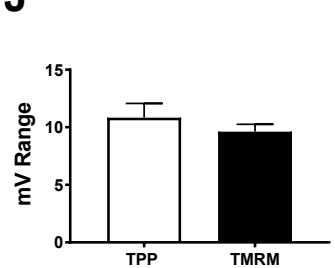
Addition	Buffer KCl [M]	mV
1	0.000375	154.16
2	0.00075	135.64
3	0.001125	124.80
4	0.001625	114.97
5	0.002125	107.80
6	0.002625	102.16

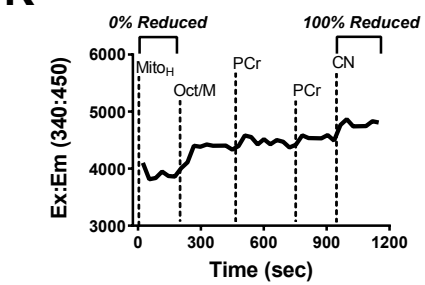




(1) 1.15μM TPP
(2) 1.2μM TPP
(3) 1.25μM TPP
(4) 1.3μM TPP
(5) 1.35μM TPP
(6) 150μg Mitochondria
(7) 6mM NaPCr
(8) 9mM NaPCr
(9) 15mM NaPCr







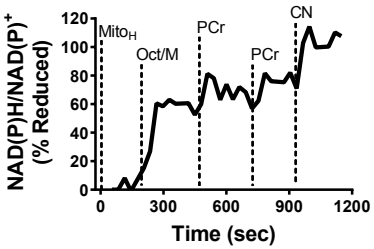


Figure S2. Approximation of mitochondrial ΔΨ via TMRM and measurement of NAD(P)H/NAD(P)$^+$ redox state under varying ATP free energies. Related to Star Methods – "Mitochondrial membrane potential (ΔΨ) and NAD(P)H/NAD(P)$^+$ Redox" and "Mitochondrial membrane potential (ΔΨ) using a TPP$^+$ selective electrode".

(A) Relationship between JO_2 and ΔG_{ATP} in isolated skeletal muscle mitochondria in the presence of TMRM (0.2 μM) or DMSO. Experiments were carried out in Buffer D, supplemented with ATP (5 mM), Cr (5 mM), PCr (3 mM) and 20U/ml CK. Assay additions were mitochondria (0.025 mg/ml), G/M and PCr x 4 (3 mM additions each). (**B**) Excitation and emission scans carried out in Buffer D, supplemented with TMRM (0.2 μM) and FCCP (5 μM) or ethanol. Mitochondria from skeletal muscle (0.1 mg/ml) were present for all experiments. (**C**) Excitation spectra calculated from the difference in fluorescence intensity between scans performed in the absence and presence of FCCP displayed in panel B (e.g., (EX Scan – EX Scan (+FCCP)) = Ex Diff). The peaks of each spectra are indicated by black arrows and the difference between them (572-551 = 21) is the quantified spectral shift experienced by TMRM as it accumulates in energized mitochondria. (**D**) Representative trace showing TMRM fluorescence at Ex/Em: 551:590 and Ex/Em: 572/590 during a CK clamp experiment in isolated heart mitochondria. (**E**) The ratio of emission intensities calculated from the experiment depicted in panel D recorded at excitation wavelengths of 572 and 551. (**F**) Representative trace displaying a ΔΨ standard curve whereby the TMRM fluorescence ratio (572/551) was plotted against time. Protocol additions were heart mitochondria (Mito, 0.1 mg/ml), valinomycin (Valino, 40 ng/ml), and KCl (indicated by numbered black arrows). The table below indicates the extra-mitochondrial [KCl] at each titration step, as well as the calculated ΔΨ according to the Nernst equation. (**G**) TMRM fluorescence ratio (572/551) plotted against the calculated ΔΨ (-mV). (**H**) Representative trace showing ΔΨ assessment using a TPP$^+$ selective electrode during a phosphocreatine titration experiment. (**I**) Relationship between ΔΨ and ΔG_{ATP} in isolated skeletal muscle mitochondria energized with Pyr/M. Membrane potential was assessed using either TMRM or a TPP$^+$ selective electrode. (**J**) Change in ΔΨ, expressed in mVs, during the phosphocreatine titration experiments depicted in panel I using both experimental methodologies. (**K**) Representative trace showing NAD(P)H auto-fluorescence (Ex/Em: 340/450) during a CK clamp experiment in isolated heart mitochondria. The annotated sections of the trace utilized to calculate % Reduction correspond to isolated mitochondria devoid of substrates (0% Reduced) and the addition of cyanide (100% Reduced). (**L**) The % Reduction in the NAD(P)H/NAD(P)$^+$ redox state calculated from the experiment depicted in panel K. (**A, G-J**) Data are mean ± SEM. Differences between groups were analyzed by 2-tailed unpaired t-tests. N=3-4/group, where "N" represents 3-4 biological replicates (i.e., mitochondrial preparations).

Supplemental Figure 3.

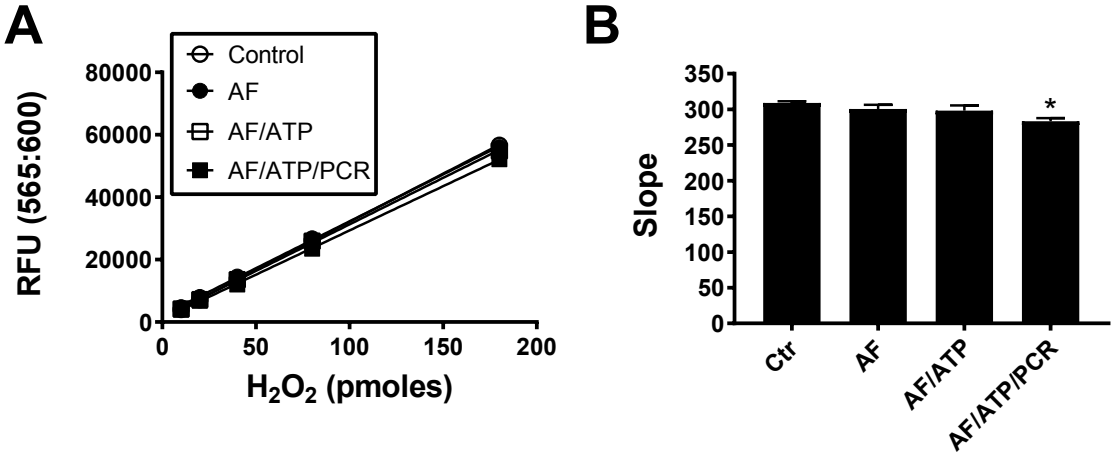


Figure S3. Related to Star Methods – "Mitochondrial $J_{H_2O_2}$ Emission".
(**A**) Resorufin fluorescence plotted against H_2O_2 in pmoles during H_2O_2 standard curve experiments performed in the presence of vehicle alone (Control), AF (0.1 μM), AF plus ATP (AF/ATP; 0.1 μM/5mM) and AF plus ATP and PCr (AF/ATP/PCR; 0.1 μM/5mM/9mM). (**B**) Calculated slopes from the experiments depicted in panel A. Data are mean ± SEM. Differences between groups were analyzed by 2-tailed unpaired t-tests comparing each group to the control (Ctr). *P<0.05. N=3/group, where "N" represents 3 biological replicates (i.e., mitochondrial preparations).

Supplemental Figure 4.

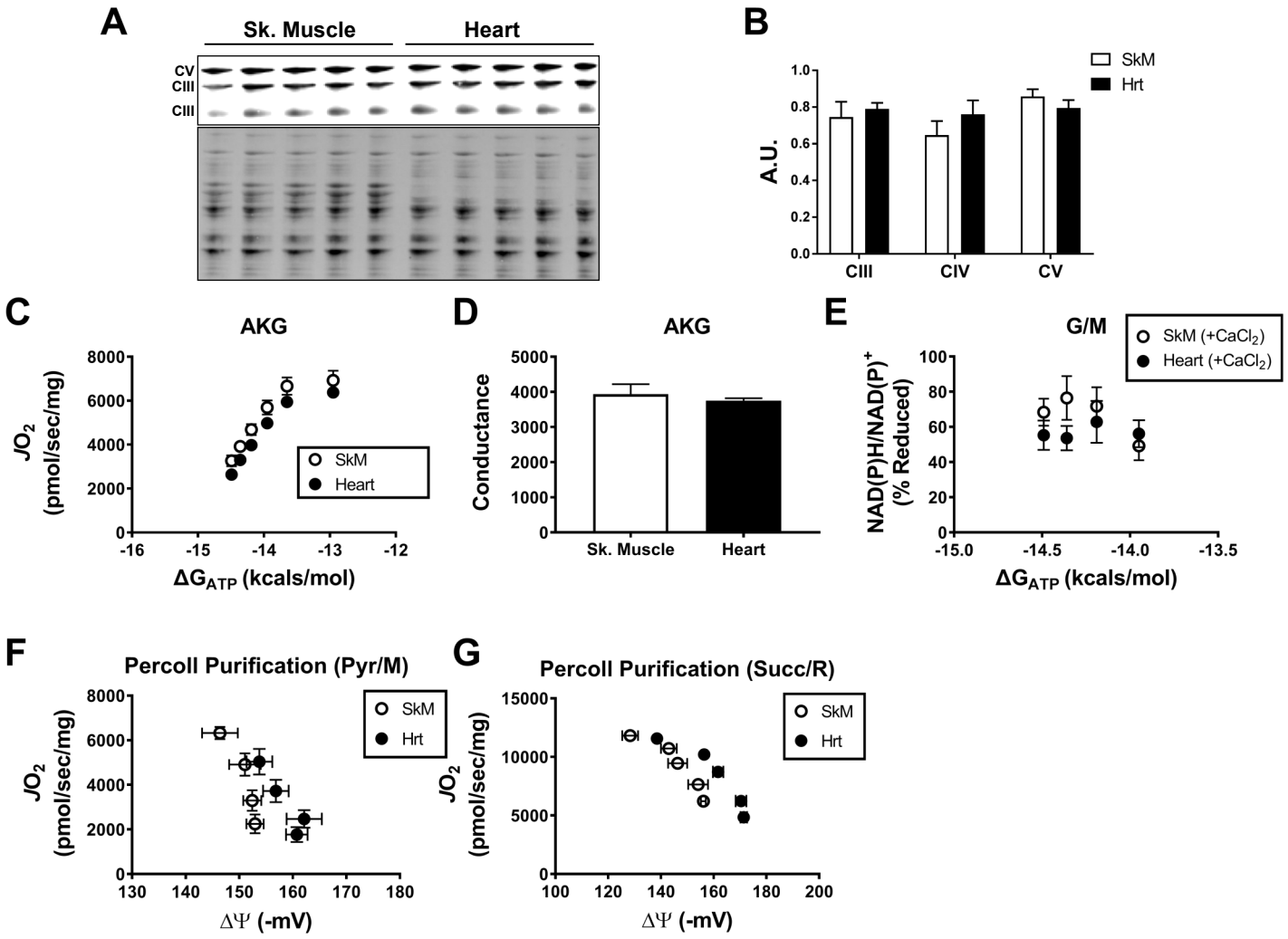


Figure S4. Related to Figures 4 and 6.

(A) Immunoblot analysis of CIII, CIV and CV in skeletal muscle and heart isolated mitochondria lysates. (**B**) Quantification of the blots depicted in panel A. (C) Relationship between mitochondrial JO_2 versus ATP free energy (ΔG_{ATP}) in mitochondria energized with AKG (10 mM). (**D**) Calculated slopes from the linear portions of the data depicted in panel A. (**E**) Relationship between mitochondrial NAD(P)H/NAD(P)$^+$ redox versus ATP free energy (ΔG_{ATP}) in mitochondria energized with G/M in the presence of 0.6 mM $CaCl_2$ (free Ca^+ ~ 500nM). (**F-G**) Mitochondria JO_2 plotted against $\Delta\Psi$ in the presence of Pyr/M (**F**) and Succ/Rot (**G**) in percoll purified mitochondria. Data are mean ± SEM. Differences between groups were analyzed by 2-tailed unpaired t-tests. (**A-F**) N=4-6 individual mice/group, (**G**) N=2 individual mice/group.

Supplemental Figure 5.

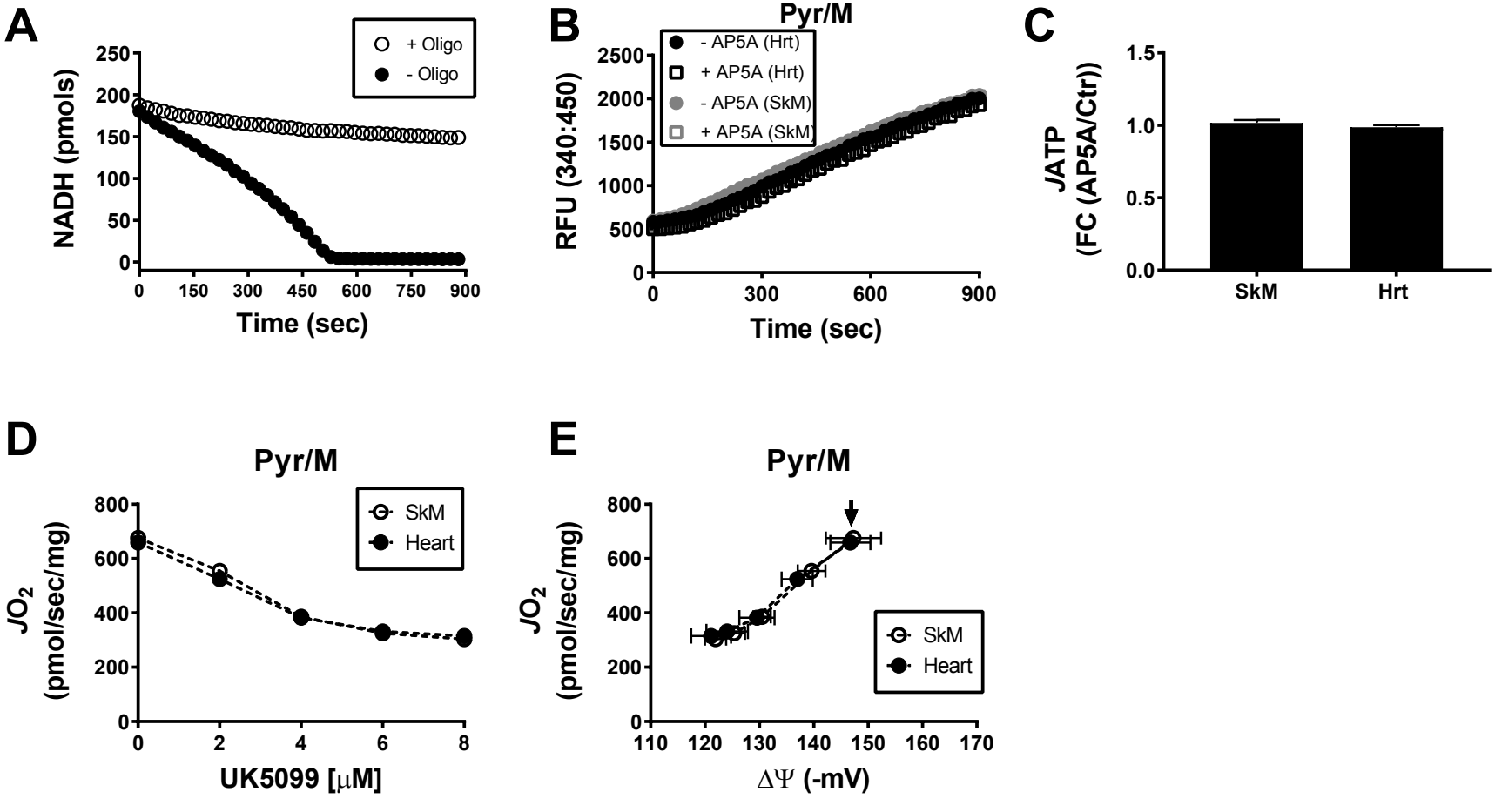


Figure S5. Related to Figure 6 and Star Methods – "*J*ATP Synthesis".
(**A**) Representative trace from a CV activity assay performed in isolated mitochondria from skeletal muscle in the absence (- Oligo) and presence (+ Oligo) of oligomycin (5 μM). **(B)** Representative trace from a *J*ATP synthesis assay performed in the presence and absence of AP5A. **(C)** Effect of AP5A on *J*ATP in skeletal muscle and heart mitochondria, expressed as fold change (FC). **(D)** Oxygen consumption in mitochondria energized with Pyr/M in the presence of oligomycin following sequential additions of the pyruvate carrier inhibitor UK5099 to titrate redox state and ΔΨ. **(E)** Relationship between proton leak and ΔΨ assessed in the presence of oligomycin and Pyr/M suggests similar proton leak rates between the two groups. Black arrow indicates leak rates at the highest common ΔΨ. Data are mean ± SEM. Differences between groups were analyzed by 2-tailed unpaired t-tests. N=4-6 individual mice/group.