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Supporting Online Material for

Function of Mitochondrial Stat3 in Cellular Respiration

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Published 8 January 2008 on *Science* Express
DOI: 10.1126/science.1164551

This PDF file includes:

Materials and Methods:

Mice: Mice homozygous for a Stat3 allele with two loxP sites in introns 17 and 20, and heterozygous mice that express α-myosin heavy chain (α-MHC)-Cre transgene were used for these experiments (*1*). Cardiac-specific Stat3-deficient mice (Stat3-/-) were created as described (*1*). Mice homozygous for floxed Stat3 and heterozygous for Cre recombinase were used as the Stat3-/- mice, and mice homozygous for floxed Stat3 and homozygous-null for Cre recombinase were used as littermate wild-type controls.

Cell Culture: Stat3-/- pro-B cells were isolated from $Stat3^{flox}$ mice crossed with Tie2-Cre mice to generate animals that do not express Stat3 in pro-B cells (*2*). Primary IL-7-dependent progenitor B (pro-B) cells were isolated from the bone marrows of Stat3+/+ and Stat3-/- mice as described (*3*). Briefly, the femurs were isolated from Stat3+/+ and Stat3-/- mice and the bone marrow cells were collected into Opti-MEM I medium (Invitrogen Corporation) containing 10% FBS, 1% Penicillin-Streptomycin, 5μM 2-Mercaptoethanol, and 2ng/mL of murine recombinant IL-7 (Biodesign International). The pro-B cells were selected in the presence of IL-7 for 10 days.

Antibodies and Reagents: Mouse monoclonal Stat3-specific antibodies were purchased from BD Transduction Laboratories and Cell Signaling. Stat3 and calreticulin polyclonal antibodies were from Santa Cruz Biotechnology. All were used at a final dilution of 1:1000.

Mouse monoclonal alpha-tubulin and porin antibodies were purchased from Oncogene and Calbiochem, respectively and used at a 1:2500 dilution. Goat polyclonal Ku-70 antibodies and rabbit polyclonal Grim-19 antibodies were purchased from Santa Cruz and Novus Biologicals, respectively, and used at a final dilution of 1:1000. Complex I

Immunocapture monoclonal antibody, NDUFA9, NDUFS3, Complex II subunit Fp,Complex III Core Protein 2, Complex IV subunit 1, Complex V α subunit and COX-I antibodies were purchased from Mito Sciences. Murine IFN β was kindly provided by BiogenIdec Inc. All other reagents were obtained from Sigma-Aldrich Chemical Co.

Construction of retroviral vectors: Murine stem cell virus retroviral vectors (MSCV-IRES-GFP), that harbors a GFP-coding sequence under the control of an internal ribosomal entry site (IRES) containing cDNAs for wild type Stat3a (MSCV-IRES-GFP-WT Stat3a), were kindly provided by Dr. Daniel Link, Washington University School of Medicine, St. Louis, MO (*4*). The mitochondrial localization sequence (MLS) from cytochrome c oxidase subunit VIII containing Nhe I and EcoR V restriction sites on 5' and 3' ends, respectively was produced as a PCR product using the following primers: Forward 5' CGC CGC TAG CAA GCT TGT GAC CAT GTC CGT CCT GAC GCC GCT GCT GCT GCG GGG CTT GAC AGG CTC GGC CCG GCG GCT CCC AGT GCC GCG CGC CAA GAT CCA TTC GTT GGG AT 3' and Reverse 5' ATC CCA ACG AAT GGA TCT TGG C 3'. The MLS sequence was cloned into the plasmid pEF4/Myc-His A (Invitrogen) digested with Nhe I and EcoR V. The cDNA sequence corresponding to the murine Stat3a protein was amplified from a pEF-BOS vector containing murine Stat3a using the following primers containing Not I and Spe I restriction sites on their 5' and 3' ends respectively: 5' TAG TTC ATG CGG CCG CAA TGG CTC AGT GGA ACC AGC TGC AG 3' (forward primer) and 5' CGA TAG ACT AGT CAT GGG GGA GGT AGC ACA CTC CGA 3' (reverse primer) and Pfu polymerase. The Stat3a cDNA sequences generated by PCR were subcloned into pEF4/Myc-His a plasmid (Invitrogen) containing the MLS sequence (digested with Not I and Xba I, and purified by gel extraction kit (Qiagen), by ligation using T4 DNA ligase (New England Biolabs).The entire construct containing MLS Stat3 was excised from pEF4/Myc-His A vector by digesting with Nhe I

and Bgl II. The purified, excised fragments were blunt-ended using Klenow. The MSCV-IRES-GFP vector was digested with Xho I restriction enzyme and also blunted. Blunt-ended and dephosphorylated MSCV-IRES-GFP vector was ligated with blunt-ended MLS Stat3a cDNA. Constructs were confirmed by DNA sequencing. Using site-directed mutagenesis kit (Stratagene), a point mutation was created from Tyr705 to Phe of Stat3a in MSCV-IRES-GFP-MLS Stat3a using the following primers – 5' CCA GGT AGT GCT GCC CCG TTC CTG AAG ACC AAG TTC ATC 3' (forward primer) and 5' GAT GAA CTT GGT CTT CAG GAA CGG GGC AGC ACT ACC TGG 3' (reverse primer). To generate Stat3 Y705F/S727A and Stat3 Y705F/S727D double mutants containing mutations of Ser727 to Ala and Asp, respectively, we used the following sets of primers – 5' ACC TGC CGA TGG CAC CCC GCA CTT 3' (forward primer) and 5' AAG TGC GGG GTG CCA TCG GCA GGT 3' (reverse primer) for the Ala mutation, and 5' ACC TGC CGA TGG ATC CCC GCA CTT 3' (forward primer) and 5' AAG TGC GGG GAT CCA TCG GCA GGT 3' (reverse primer) for the Asp mutation. Additionally, there were two glutamates (Glu434 and Glu435) within the DNA binding domain mutated to Ala to make a dominant negative mutant of MLS Stat3 E453A/E454A**.** Those two point mutations were created using the following primers 5' TTG ATC GTG ACT GCG GCG CTG CAC CT 3' (forward primer) and 5' GAT CAG GTG CAG CGC CGC AGT CAC GA 3' (reverse primer). Stat3CA was cloned into MSCV-IRES-GFP as described (*4*).

Transfection and Infection: The empty vector (MSCV-IRES-GFP), the vectors encoding wild type Stat3a and different MLS mutants of Stat3a were transfected into Phoenix packaging cells using Fugene transfection reagent (Roche). Virus-containing medium was collected at 48 and 72 h and used to infect Stat3-/- pro-B cells (*5*). The cells expressing GFP, following 7 to 10 days of infection, were sorted by FACS to 100% purity and used for experiments.

Isolation of mitochondria and preparation of cytosolic and mitochondrial lysates: Intact mitochondria were isolated from murine hearts and primary bone marrow-derived pro-B cells using differential centrifugation as described (*6*). Hearts were excised from 2-4 month old, Stat3-/- or wild type mice and collected into 10 volumes of ice-cold buffer A (20mM HEPES pH 7.4, 250mM sucrose, 1mM EDTA, 1mM PMSF, 5µg/ml aprotinin, 1µg/ml leupeptin, 25mM NaF, 1mM sodium vanadate). Alternatively, pro-B cells were harvested, washed once with cold PBS, and resuspended in ice-cold buffer A.

Both the hearts and pro-B cells were then dounce homogenized and unbroken cells and nuclei were removed by centrifugation at 500 x g for 5 min at 4°C. The supernatants were further centrifuged at 7700 x g for 10 min at 4°C to obtain the crude mitochondrial fraction. The supernatants resulting from the above step were subjected to a high-speed centrifugation at 100,000 x g for 1 h at 4°C to obtain the cytosolic fraction. The crude mitochondrial pellet was resuspended in cold buffer A and layered on the top of a 2.5 M sucrose-percoll gradient and centrifuged at 46,000 x g for 45 min at 4°C to separate the pure mitochondrial fraction. The mitochondria were collected and washed twice with buffer A at 7700 x g for 10 min at 4°C and lysed in presence of cold buffer A plus 1% Chaps for 30 min at 4°C. The lysates were then centrifuged at 15,000 x g for 10 min at 4°C to remove insoluble debris. Protein concentration of both mitochondrial and cytosolic lysates was determined using the Bradford dye-binding protein assay (Bio-Rad).

Preparation of nuclear and whole cell extracts: For preparing nuclear extracts, pro-B cells were resuspended in 1 mL of cold nuclear isolation buffer (20mM Hepes pH 7.0, 10mM KCl, 10mM $MgCl_2$, 1mM sodium orthovanadate, 0.5mM DTT, 0.25mM PMSF, 20% glycerol, 0.1% NP40, 5µg/mL aprotinin, 1µg/mL leupeptin) and dounce

homogenized until >90% of the cells were trypan blue-positive**.** Isolation of nuclear and cytosolic fractions and preparation of whole cell extracts were performed as described (*7*).

Western blot analysis: Whole cell, cytosolic, nuclear, or mitochondrial lysates (30 μg each) were resolved on 8% or 15% SDS-PAGE gels, transferred to an Immobilon-P PVDF membrane (Millipore), and the membrane was incubated for 30 min with casein blocker (GE Healthcare, Piscataway, NJ, USA). The blots were incubated overnight at 4ºC with the primary antibody diluted in TBS-Tween-20 buffer (20mM Tris-HCl pH 7.5, 500mM NaCl, 0.05% Tween 20). Following primary antibody incubation, the blots were washed and incubated with 1:5000 dilution of the horseradish peroxidase-conjugated secondary antibody (Zymed) and developed using ECL (GE Healthcare).

Immunoprecipitation: Mitochondria were isolated from pro-B cells using differential centrifugation as described (*6*). Isolated mitochondria were lysed in lysis buffer (20mM Hepes pH 7.4, 1% Triton X-100, 250mM sucrose, 1mM EDTA, 1mM PMSF, 5μg/mL aprotinin, 1μg/ml leupeptin, 25mM NaF, 1mM sodium vanadate), and 1 mg of lysates were immunoprecipitated overnight with 2.5 μL of Complex I Immunocapture monoclonal antibody or isotyped match control (Mito Sciences). Beads were washed four times with washing buffer (20mM HEPES pH 7.4, 0.05% Triton X-100, 250mM sucrose, 1mM EDTA, 1mM PMSF, 5μg/ml aprotinin, 1μg/ml leupeptin, 25mM NaF, 1mM sodium vanadate). Total mitochondrial lysates or immunoprecipitates were separated by SDS-PAGE and immunobloted with rabbit C-terminal anti-Stat3 and other antibodies (Cell Signalling/MitoSciences) and mouse anti Grim-19 antibodies (Novus Biologicals) as described above.

Isolation of total mRNA, cDNA synthesis, and real-time PCR analysis for mRNA quantification: mRNA was prepared using RNAzol B, (Teltest Inc), from untreated pro-B cells or those treated with murine IFN β (1000 U/ml) for 1 h. 2μg of total mRNA from the respective samples was used to generate cDNA. SOCS3 and ADAR expression were determined by real-time PCR using SYBR Green PCR master mix kit (Applied Biosystems). β-actin RNA concentrations were used as an internal control. The following primers were used to analyze the β-actin cDNA levels – 5'-ACC AGT TCG CCA TGG ATG AC-3' (forward) and 5'-TGC CGG AGC CGT TGT C-3' (reverse). The primers used to analyze the cDNAs for SOCS-3 and ADAR were obtained from SuperArray Bioscience Corporation.

Isolation of total DNA and real-time PCR analysis for mtDNA quantification:

Total DNA was extracted from 5 x 10^7 cells from wild type and Stat 3-/- pro-B cells. DNA levels of mitochondrial-encoded genes (mtDNA): NADH:ubiquinone oxidoreductase subunit 1 (ND 1), ATP synthase subunit 6 (ATPase 6), cytochrome c oxidase subunit 1 (COX-1), cytochrome c oxidase subunit 2 (COX-2), and β-actin were analyzed by quantitative PCR using specific primers. Real-time PCR analysis was carried out using SYBR Green PCR master mix kit (Applied Biosystems) as per the manufacturer's guidelines. The following primers were used to analyze the β-actin DNA levels – 5' ACC AGT TCG CCA TGG ATG AC 3' (forward) and 5' TGC CGG AGC CGT TGT C 3' (reverse), ND1: CAG GAT GAG CCT CAA ACT CCA 3' (forward) and 5' CGG CTC GTA AAG CTC CGA 3' (reverse), ATPase 6: 5' ATT CCC ATC CTC AAA ACG CC 3' (forward) and 5' TGT TGG AAA GAA TGG AGA CGG T 3' (reverse), COX-1: 5' TCG CAA TTC CTA CCG GTG TC 3' (forward) and 5' CGT GTA GGG TTG CAA GTC AGC

3’ (reverse), and COX-2: 5’ GAG CAG TCC CCT CCC TAG GA 3’ (forward) and 5’ TGA TTT AGT CGG CCT GGG AT 3’ (reverse). The samples were assayed and analyzed using 7300 RT-PCR system (Applied Biosystems).

Preparation of crude mitochondrial fractions from pro-B cells and mouse hearts for measuring mitochondrial respiration: Mitochondria were isolated from pro-B cells (*6*) and murine hearts (*8, 9*) using differential centrifugation. Hearts were excised from 2-4 months old, Stat3-/- or Stat3 $^{flox/flox}$ wild type mice and collected into 10 volumes of ice-cold buffer A (20mM HEPES pH 7.4, 250mM sucrose, 1mM EDTA, 1mM PMSF, 5µg/mL aprotinin, 1µg/mL leupeptin). Alternatively, cells were harvested by centrifugation, washed once with cold PBS, and resuspended in ice-cold buffer A. Both the hearts and pro-B cells were then dounce homogenized and unbroken cells and nuclei were removed by centrifuging the lysates at 500 x g for 5 min at 4°C. The supernatants were collected and further centrifuged at 7700 x g for 10 min at 4°C to obtain the crude mitochondrial fraction. The mitochondria were washed twice with buffer A and finally resuspended in the same buffer A. Mitochondria were used to assess the function of the electron transport chain by oxygen consumption assays (OXPHOS and oxidase assays) and measuring individual complex activities of the electron transfer chain (ETC assays). The protein concentration of mitochondrial preparations was determined using the Bradford dye-binding protein assay (Biorad).

OXPHOS assays of mitochondria from pro-B cells and mouse hearts: Oxygen consumption by intact mitochondria was measured polarographically in a chamber equipped with a Clark-type oxygen electrode (Strathkelvin Instruments Ltd.) at 30°C. The assays were done as described (*10, 11*). State 3 and State 4 respiration were defined

and calculated as ADP-stimulated and ADP-limited respiration, respectively. Respiratory control ratios (RCR - ratio of State 3 to State 4 respiration) were calculated.

Oxidase assays of mitochondria from pro-B cells and mouse hearts: Mitochondrial oxidase assays were carried out using frozen and thawed mitochondria and were performed at 30°C using the oxygen electrode (*10, 11*). Substrates for these assays included NADH and DHQ. Activities were determined in the absence or presence of specific inhibitors and were calculated as the difference of uninhibited minus inhibited rates.

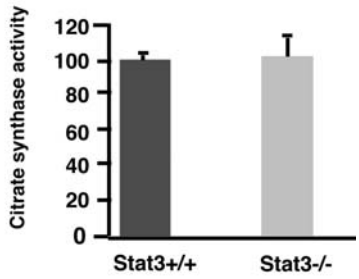
Measuring individual complex enzyme activities of the electron transfer chain (ETC assays) of mitochondria from pro-B cells and mouse hearts: Enzyme activities were measured using solubilized mitochondria (250 μg of mitochondrial protein solubilized in 0.1 M potassium phosphate buffer pH 7.2 containing 1% cholate, to a final volume of 250 μl).

NADH:duroqinone oxidoreductase (complex I) (68), Succinate:2,6- dichlorophenol (DCPIP) oxidoreductase (complex II), Ubiquinol:ferricytochrome c oxidoreductase (complex III) and Ferrocytochrome c:oxygen oxidoreductase (complex IV): Activities were measured spectrophotometrically as described (*10, 11*).

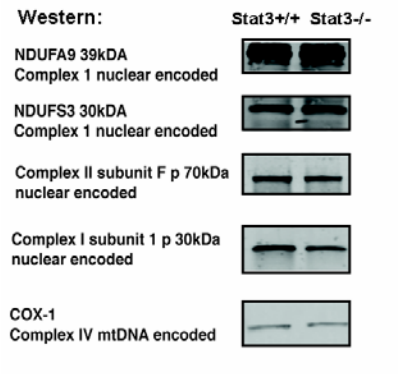
Citrate synthase activity: Activities were measured spectrophotometrically as described (*8*).

Statistical analysis: The data were analyzed using a student's t-test. A p value greater than 0.05 was considered to be statistically significant.

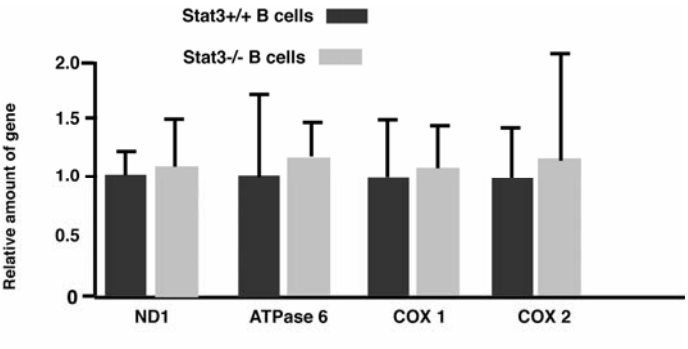
Supplement Fig.1A.



Supplement Fig.1B.



Supplement Fig.1C.



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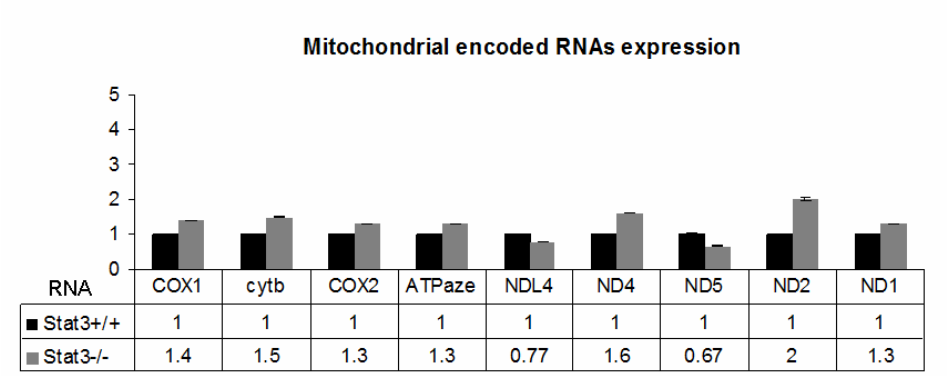
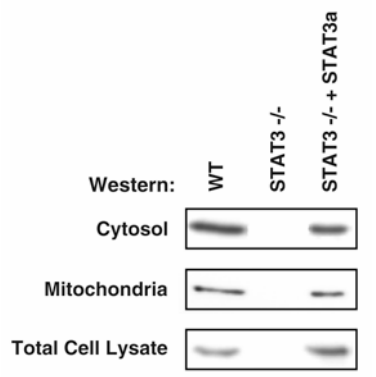


RNA	COX1	cytb	COX2	ATPaze	NDL4	ND4	ND5	ND2	ND1
Stat3+/+	1	1	1	1	1	1	1	1	1
Stat3-/-	1.4	1.5	1.3	1.3	0.77	1.6	0.67	2	1.3

Supplement Fig.2A.



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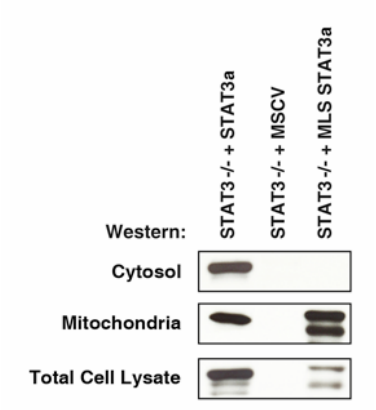
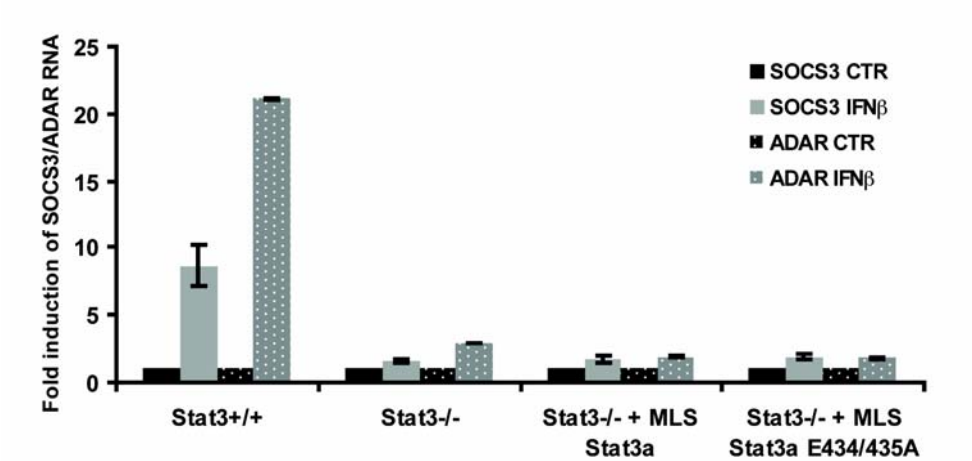
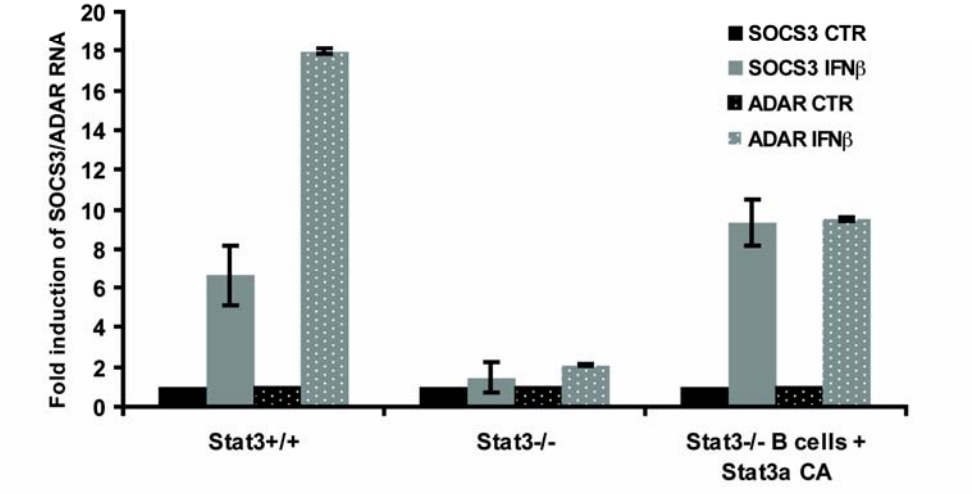
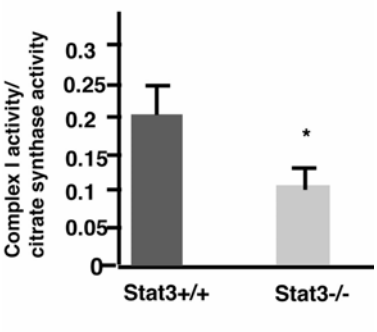


Supplement Fig.3.





Supplement Fig.4A.



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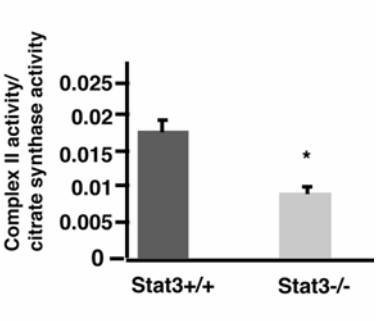

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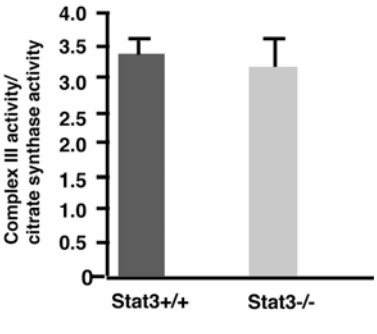

Supplemental Figures

Supplemental Figure 1. Mitochondrial content is not altered in Stat3-/- pro-B cells. A) Citrate synthase activity in wild type and Stat3-/- cells. Equal numbers of wild type and Stat3-/- cells were harvested and the activity of citrate synthase was analyzed. The citrate synthase activity in Stat3-/- pro-B cells is expressed as a percentage relative to wild type cells which were given a value of 100, and are represented as mean ± SD (%), n=3 independent experiments and each experiment was performed in duplicate. **B)**

Expression of components of the ETC are similar in wild type (Stat3+/+) and Stat3-/- pro B cells. Total cell lysates were prepared fropm equal amounts of WT and Stat3-/- cells, separated by SDS-PAGE and probed with the indicated antisera. **C) Real-time quantitative PCR analysis of mitochondrial DNA-encoded genes in wild type and Stat3-/- pro-B cells.** Total DNA was isolated from 5×10^6 cells and analyzed for levels of mitochondrial genes by real-time quantitative PCR. DNA levels of actin were used as an internal control to normalize the amount of mitochondrial genes. Results are expressed as relative levels of mitochondrial genes in Stat3-/- cells compared to wild type cells. The values represent means fold increase $\pm$ SD, n=3 independent experiments and each experiment was performed in duplicate. **D) The amounts of mitochondrial encoded RNAs are the same in wild type (Stat3+/+) and Stat3-/- cells. RNA was prepared from equal amounts of Stat3+/+ and Stat3-/- cells.** The relative amount of mitochondrial encoded RNAs was analyzed by using qPCR and normalized to the amount of β-actin in the sample. The amount of a given RNA in Stat3+/+ cells was set given a value of 1.

Supplemental Figure 2. Stat3-/- cells reconstituted with Stat3a or Stat3a that contains a mitochondrial localization sequence (MLS Stat3a). Stat3-/- pro-B cells expressing Stat3a **(A)** or MLS Stat3a, **(B)** were fractionated into mitochondrial (mitochondria), cytosolic (cytosol), and nuclear (nuclei), and total cell lysates were also examined. The immunoblots were probed for Stat3. There appear to be two bands in the mitochondrial fraction of MLS Stat3. This finding is not seen in all preparations. However, it has been reported that Stat3 can be proteolytically cleaved (*12, 13*).

Supplemental Figure 3. Separation of nuclear and mitochondrial function of Stat3. A). Activation of SOCS3 and ADAR1 mRNA Expression by IFN β is absent in cells expressing Stat3a with a mitochondria localization sequence and/or a mutation in its DNA binding domain. Pro-B cells were incubated with or without IFN β (1000 units/ml) for 1 h prior to preparation of mRNA. SOCS3 and ADAR1 expression was measured by RT-PCR and normalized to the amount of β-actin in the sample. CTR, Control. **B) Stat3-/- cells expressing Stat3CA show normal IFNβ stimulated SOCS3 and ADAR1 RNAs.** The conditions of the experiment are described above.

Supplemental Figure 4. Enzymatic activities of ETC complexes I and II in heart mitochondria from wild type and Stat3-/-mice: complex I (A), complex II (B), and complex III (C). Cholate-solubilized mitochondria were used for these measurements. Enzyme activities were determined spectrophotometrically as described in Materials and Methods and are expressed as miliunits (nmol/min) per mg of mitochondrial protein and were normalized to citrate synthase activity. Results are presented as mean ± SD. Significant differences (*p<0.05) between Stat3-/- and wild type mice are shown.

Electron transport chain complexes required	Wild type 2mM ADP	Stat3-/- 2mM ADP
Complexes I, III, IV 10mM pyruvate 5mM malate	0.11 ± 0.01 (N=5)	0.030 ± 0.01 (N=5) p= 0.00002
Complexes II, III, IV 20mM succinate	0.18 ± 0.07 (N=6)	0.07 ± 0.02 (N=7) p=0.001

Supplemental Table 1. Maximal rates of State 3 respiration in isolated heart mitochondria from wild type and Stat3-/- mice incubated with pyruvate/malate or succinate. Samples contained 10mM pyruvate and 5mM malate, or 20mM succinate, and 7.5 μM rotenone. Results, expressed as nAtom O/min/mg of mitochondrial protein, were normalized to citrate synthase activity and are presented as mean ± SD. P values for the comparisons are listed in the table. Significant differences between wild type and Stat3-/- mitochondria are noted in the table.

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