The role of deimination in ATP5b mRNA transport in a transgenic mouse model of multiple sclerosis

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Supplementary Materials and Methods

PC 12 cell culture and siRNA treatment

PC12 cells were purchased from ATCC (Manassas, VA, cat. CRL-1721). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Cellgro, Manassas, VA), supplemented with 10% horse serum (ATCC), 5% fetal bovine serum (Cellgro) and 1% antibiotic-antimycotic solution (Cellgro). The siRNA experiments were performed using siRNA against peptidyl arginine deiminase 2 and peptidyl arginine deiminase 4 (PAD2 and PAD4) sequences [Stealth Select RNAi for rat (Rattus norvegicus) PAD2 (catalog numbers, Oligo id#, RSS309706, RSS309707, RSS309705) and for PAD4 (catalog numbers, Oligo id#, RSS309711, RSS309713, RSS309712) from Invitrogen]. PAD2 and PAD4 are major cytosolic and nuclear deiminases in the neuronal systems that converts protein-bound arginines into protein-bound citrulline. Control siRNA (Invitrogen) unrelated to any known mammalian sequence was also used. Another set of siRNA experiments were performed using siRNA against REF (catalog numbers, A-108153-01; Thermo Scientific Dharnacon, Inc. Lafayette, CO). The siRNA was transfected using Lipofectamine 2000 (invitrogen), following the manufacturer's instructions with minor modifications. Briefly, 10^6 were collected for each transfection. All siRNA used here were carefully evaluated and were found to down regulate about 80% of PAD2, PAD4, and REF mRNA expression. About 10^6 siRNA treated cells were used for each single ATP synthase activity measurement. Cells were suspended in DMEM and plated onto 35-mm Petri-dishes coated with poly-D-lysine (Sigma Chemical Co., St. Louis, MO) after transfection, and grown with differentiation medium containing 1% horse serum (ATCC), 1% antibiotic-antimycotic solution (Cellgro) and 100ng/ml nerve growth factor (NGF) (Sigma Chemical Co.). The cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every three days.
Mouse model. All animal experiments were approved by Institutional Animal Care and Use Committee and performed adhering to ARVO (The Association for Research in Vision and Ophthalmology) statement of animal use in vision research. Breeding pairs of ND4 mice were obtained from Dr. Mario Moscarello (Hospital for Sick Children, Toronto, Ontario, Canada), and a pathogen-free line was derived via embryo transfer performed at the Charles River Laboratory (Wilmington, MA). The ND4 and CD1 mice (wild-type [WT]) were housed in covered cages and maintained in a controlled environment.

Isolation of brain mitochondria. Brain mitochondria were isolated following a procedure previously described (Dunkley et al., 1988). All mitochondrial respiration experiments were performed in synaptosomes permeabilized with 0.007% digitonin (Dave et al., 2008). Generally, mice were decapitated under isoflurane anesthesia. Brains and spinal cords were removed immediately and immersed into cold (4°C) isolation medium. Isolation medium consisted of 250 mM sucrose, 1 mg/ml bovine serum albumin (BSA, EMD, Gibbstown, NJ), 1.0 mmol EDTA, and 0.25mM dithiothreitol (DTT) pH 7.4. Tissue was minced with a pair of scissors and rinsed thoroughly with the isolation medium. The minced tissue was suspended in isolation buffer, and then was homogenized with a hand operated glass Teflon homogenizer by seven up-and-down strokes. The homogenate was diluted to a final concentration of 10% (W/V) and was centrifuged at 500 X g for 5 minutes in Sorvall RC5 centrifuge. The supernatant was collected from 3 mice brains and spinal cords. The pooled supernatant was layered on the Percoll gradient. The gradients were prepared in 12 ml polycarbonate tubes and consisted of 2 ml each of 23, 15, 10, and 3% (V/V) Percoll. The gradients were centrifuged at 32,500 X g for 5 minutes. The nonsynaptic mitochondrial pellet was collected from the bottom of the 23% Percoll layer; the synaptic mitochondria were collected at interface of 23 and 15% and 15 and 10% layers and
combined. The collected mitochondria were washed once with isolation media, using 12 ml polycarbonate tubes, by centrifugation at 15,000 X g for 10 minutes. The resulting non-synaptic and synaptic mitochondrial pellets from different fractions were re-suspended in 0.32 mol/l sucrose to give approximately 4-7mg/ml.

**Respiration studies in isolated mitochondria.** Substrate oxidation rates and phosphorylating capacities of isolated mitochondria were determined by polarograph following the procedure previously described (Dave et al., 2001; Hofhaus et al., 1996) Oxygen consumption was measured in 300 µl 25 mM Tris–HCl (pH 7.4) and 10 mM potassium phosphate buffer (pH 7.4) containing 150 mM sucrose by a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England), which sits in 0.25 mL-capacity incubation microassay chamber with a water jacket (30°C) and. The buffer inside of the chamber was stirred continuously using an electromagnetic bar stirrer at 40 rpm. The oxygen electrode was connected to a computer, and oxygraphs were recorded using oxygraph software (Hansatech Instruments). For respiration studies, synaptosomes were permeabilized with 0.007% digitonin.

The mitochondrial respiratory control index (RCI) and ADP–O ratios were measured in presence of 5 mM pyruvate and 2.5 mM malate. For each experiment, approximately 0.03 to 0.05 mg mitochondrial protein was added into the assay. The RCI is defined as the ratio of the respiratory rate in presence (state 3) and absence of ADP (state 4) (Chance and Williams, 1956). The ADP–O ratios were measured polarographically, as described earlier (Chance and Williams, 1956). In brief, ADP–O ratio is defined as moles of ADP phosphorylated per moles of oxygen consumed (slope of state 3 over the time required for total consumption of the ADP added to the buffer).
Measurement of mitochondrial complex activity. The experiments were performed following the procedure previously described with modification (Dave et al., 2008; Rustin et al., 1994). All reactions were carried out in total volume of 1ml at 37°C.

Measurements of rotenone-sensitive NADH-decylubiquinone oxidoreductase (complex I). Mitochondrial protein (about 60 µg) were suspended in 10mM Tris-HCl buffer (pH8.0) containing 1mg/ml BSA were incubated at 37°C water bath for 3 min to make the mitochondrial internal membrane permeable before adding the substrates. Decylubiquinone (acceptor) and NADH (donor) were added to make the final concentration at 50 µM and 0.8 mM respectively. The absorbance at 340nm was recorded for 2 min.

Measurements of succinated phenazine methosulfate dichlorophenol indophenol reductase (complex II). Mitochondrial protein (about 30 µg) were suspended in 10mM KH2PO4 (pH7.8) containing 2mM EDTA and 1mg/ml BSA with methyl phenazine methosulfate (1mM), rotenone (3 µM), ATP (0.2 mM), and KCN (0.3 mM) (Barrientos et al., 1998). The absorbance was recorded at 600nm for 2min after adding acceptor 2,6-dichlorophenol indophenols (100 µM) and donor succinate (16mM). 10mM malonate was used as inhibitor.

Measurements of decylubiquinone cytochrome c reductase (complex III). Mitochondrial protein (about 30 µg) were suspended in 10mM KH2PO4 (pH7.8) containing 2mM EDTA and 1mg/ml BSA with succinate (10 mM), rotenone(3 µM), ATP (0.2 mM), and potassium cyanide (0.3 mM). The absorbance was recorded at 550nm for 2min after adding acceptor cytochrome c (oxidized) (40 µM) and donor duroquinol (50mM). The initial rate of cytochorome c reduction was used for the calculation of activity.
Measurement of cytochrome c oxidase (complex IV). Mitochondrial protein (about 15 µg) were suspended in 10 mM phosphate buffer (pH 6.5), 0.3µM sucrose, 1 mg/ml BSA and permeabilized the external mitochondrial membrane permeable with 2.5mM lauryl maltoside. The absorbance was recorded at 550nm for 2min after adding donor cytochrome c (reduced) (40 µM). The initial rate of cytochrome c reduction was used for the calculation of activity.

Immunoprecipitation (IP). All procedures were performed under RNase free conditions for the following experiments. Retinal cytosolic lysates and lysates prepared from mitochondria isolated from mouse brain were subjected to immunoprecipitation using anti-REF and anti-citrulline (Upstate, Lake Placid, NY) antibodies. Briefly, IP was carried out as follows: about 67 µg sepharose A overnight swelled beads suspended in 200 µl of 50 mM Sodium borate buffer pH 9.0 were incubated with 10 µg of anti-REF (ALY: catalog numbers ab6141; Abcam Inc., Cambridge, MA) or anti-ALDH1 antibody (ALDH1L1; catalog numbers ab56777; Abcam Inc.) at room temperature for an hour. The beads and antibody were cross-linked by adding dimethyl pimelimidate dihydrochloride (DMP) 10 µg three times each with 2 hour interval incubation at room temperature, and then was kept at 4 °C overnight, subsequently neutralized with 200 mM ethanolamine 200 µl, and washed with phosphate buffered saline (PBS) 1ml twice. The antibody coupled beads were then divided into two equal parts and incubated with 200 µg cytosolic or mitochondrial lysate for an hour at room temperature, respectively. The beads were washed with 500 µl of PBS twice, and eluted with 30µ1 of 100mM glycine pH 3.0 twice. The eluents were combined and divided into 2 equal amounts; one was kept at 4°C for separation on SDS-PAGE and the other was used for mRNA isolation. For the citrulline IP, cytosolic and mitochondrial lysates were pre-treated with 2, 3-butatanedione and antipyrine in acidic condition using the citrullination detection kit (cat no. 07-390; Upstate).
RNA extraction and reverse transcription PCR. As described previously, IP anti-REF experiments were carried out using mitochondrial and retinal cytosolic extracts. The eluents were collected respectively, and RNA species came out together with REF and were isolated using the miniRNA extraction kit (Stratagene Inc., La Jolla, CA) as per manufacturer’s recommended protocol. Total RNA was dissolved in DEPC-treated distilled water and converted to cDNA with the oligo dT (12-18) (Invitrogen Inc., Carlsbad, CA) following two step reactions provided by the company. For detection of mRNA species, RT-PCR of IP product derived mRNA converted cDNA was carried out with primers for Tfam, β-actin, ATP5b and ATM1 (Supplemental Table S2) under a mild PCR condition according to manufacturer’s instruction (advantage cDNA PCR kit; Clontech Inc., Mountain View, CA) and separated on a 2.0% agarose gel made with TBE buffer.

Real Time PCR. Real-time PCR was performed using an iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA) associated with the inbuilt interface software (version 2.3; Bio-Rad) using primer pairs shown in Supplemental Table S2. All PCR experiments were carried out in triplicate with a reaction volume of 25 µl, using iCycler IQ 96-well optical grade PCR plates (Bio-Rad) covered with iCycler optical-quality sealing film (Bio-Rad). Mastermix was prepared as follows (to the indicated end-concentration): 0.4 µM forward primer, 0.4 µM reverse primer and 12.5 µl of SYBR Green PCR Universal master mix (2×, Bio-Rad). Amplifications were done with the following temperature procedure: one cycle at 95°C (3 min), 35 cycles of denaturation at 95°C (30 seconds), primer annealing at 56°C (30 seconds), and one final cycle at 95°C (30 seconds). Finally, melt curve analyses were made by slowly heating the PCR mixtures from 55 to 95°C (1°C per cycle of 10s) with simultaneous measurements of the SYBR Green I signal.
intensities. Quantitation was done by using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each set of primers.

**Cloning, purification, RNA isolation and binding experiments.** REF clone (EMM1002-96824126) in plasmid pExpress1 procured from OpenBiosystems, Huntsville, AL, was subcloned in pET19 vector (Novagen, Cat. No. 69677-3). The clone was sequenced and transformed in *E. coli* BL21 cells induced using 0.1 mM IPTG at absorbance values of 0.55-0.8 at 600 nm. Recombinant his-tagged REF was purified using two rounds of Ni-NTA column (Qiagen, Valencia, CA) according to the protocol from the company. In the second round the purified product from the first round was extensively dialyzed using a 3500 MWCO membrane (Sigma Chemical Co., St. Louis) in PBS and subjected to bind to Ni-NTA column and re-purified eluting with only 100 mM imidazole. The final purified product was subjected to dialysis.

For *in vitro* deimination of recombinant REF, a recombinant PAD2 was prepared. Briefly, PAD2 clone (openbiosystems, MHS1010-7507607) was subcloned into pQE1 vector as a GST fusion (Glutathione S transferase; using ligation of GST generated by PCR amplification) DNA. The GST column purified PAD2 was incubated under deiminating conditions with dialyzed REF. The recombinant REF (100 µg) was deiminated *in vitro* using 10 µg of recombinant PAD2 and re-purified using Ni-NTA column, dialyzed and quantified using Bradford’s method.

RNA binding experiments were performed using 10 µg of purified his-tagged recombinant non-deiminated or deiminated REF incubated with about 400 µg total RNAs (isolated from 3 month old C57BL6/J mouse brain) using Triazol method, standard protocol for an hour, subsequently the mixture was incubated with 100 µl of Ni-NTA beads. The bead bound protein was loaded onto a mini column, washed with 50 volumes of binding buffer, and was eluted with the same
buffer containing 100 mM imidazole. The eluted RNA product was precipitated using carrier BSA and extracted by chloroform and subjected to microarray analysis.

**Exogenous recombinant control and deiminated REF coupled Transcription-translation of ATP5b.** A quick coupled transcription-translation system (TNT-T7, cat no. L1170; Promega Corp., Madison, WI) was used for these experiments following the manufacturer’s recommendation following suitable modification of our previously published protocol (Picciani et al., 2009). TNT quick master mix was added with 200ng of pReceiver-ATP5b plasmid (Genecopoeia Inc., Rockville, MD) with 10µg isolated mitochondria from hippocampal neurons and incubated for 90 minutes at 30°C in absence or in presence of 5 µg of recombinant non-deiminated or in vitro deiminated REF or without any REF addition. An experiment was also performed with 200ng of pReceiver-GFP plasmid and 10µg isolated mitochondria added to the TNT mix served as background for ELISA. After incubation, the mixture was centrifuged at 15,000 rpm and pellet was resuspended in 0.1% Genapol containing 125mm TrisCl buffer pH7.0 and sonicated twice for 30 seconds. The synthesized proteins the precipitated with mitochondria were immediately analyzed by direct ELISA with 1: 2000 dilution of anti-ATP5b antibody. For this purpose, a 96-well flexible PVC plate (cat no. 353912; BD Biosciences, San Jose, CA) was incubated with 100 µL of1:1 dilution in 1X phosphate buffered saline of the sample at 37°C for 1 hour. The plate was washed three times with 1X phosphate buffered saline and then blocked with 0.2% BSA at 37°C for 1 hour, an alkaline phosphatase–coupled secondary antibody and a plate reader were used for detection at 405nm.

**Blue-native polyacrylamide gel electrophoresis (BN-PAGE).** Mitochondria were isolated from CD1 and ND4 mice as describe above. Mitochondrial lysate were prepared using NativePAGE™ Sample Prep Kit (Invitrogen). Briefly, mitochondrial samples were lysed with
1% of digitonin. Mitochondrial lysates (50µg each) from wild type and ND4 mice were separated on a 3-12% gradient BN-PAGE gel (Invitrogen). The lanes were cut and incubated with a dissociating solution (1% SDS and 1% β-mercaptoethanol) for an hour (Calvaruso et al., 2008). The lanes were loaded on top of second dimension SDS-PAGE gel (Invitrogen) followed by Western blot analysis.

**Protein protection assay.** Isolated Mitochondria were resuspended in 50 mM Tris-HCl, pH 7.5, 320mM sucrose, 1 mM EDTA. The resulting mitoplasts were centrifuged at 12,000 g for 10 min at 4°C. The pellet was resuspended in 320mM sucrose, 20 mM TrisCl, pH 7.5, 1 mM EDTA and 100 µg was used for Proteinase K treatment. Control sample was added with 20mM EDTA before Proteinase K treatment. Mitoplasts were incubated with Proteinase K (60 µg, Sigma) on ice for 5-10 min and the reactions were stopped by the addition of 4 mM PMSF (Sigma). The pellets obtained after centrifugation at 12,000 g for 10 min at 4°C were dissolved in SDS-sample buffer, subjected to SDS-PAGE and analyzed by Western blot analysis.

**Mass spectrometric identification of deiminated REF.** The endogenous REF (Refbp2) from wild type and ND4 hippocampal neurons was purified using an antibody conjugated Sepharose A (0.2 ml) column followed by Mono-Q and Mono-S columns (1ml; GE Healthcare corporation, Piscataway, NJ) employing a FPLC system. Purified intact REF was subjected to mass spectrometry on a MALDI-TOF device (Voyager DE Pro, ABI Inc.) analyzed in linear mode. REF from wild type yielded m/z ratio of 23730.25 corresponding to unmodified REF and a large peak for 23737.32 corresponding to deiminated REF (7 arginines were confirmed) and REF from ND4 mice yielded only one peak with m/z ratio of 23730.28 corresponding to unmodified REF.

**Microarray experiments.** The RNA eluted from at least two independent binding experiments as describe above, each using recombinant non-deiminated and deiminated REF, was prepared to
cDNA followed by two rounds of amplification according to standard protocol. The amplified products are used for hybridization separately (four different hybridizations) using Affymatrix Mouse Genome 430 2.0 Array chip (NIH Neuroscience Microarray Consortium, Translational Genomics Research Institute (TGen), Neurogenomics Division, Phoenix, AZ). The array hybridization results were prepared and the representative mRNAs are shown in Supplemental Table S1. Microarray data has been submitted to GEO database, accession number GSE11843.

**Electrophoretic Gel Mobility Shift.** The non-radioactive light shift chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology, Rockland, IL) and 5’-biotin end-labeled oligonucleotides were used according to the manufacturer’s recommended protocol to detect RNA and protein interactions. Recombinant non-deiminated REF and in vitro deiminated recombinant REF were generated to test with different single strand DNA probes, Supplemental Table S3. Purified REF protein (10 µg) was incubated with 0.25 pM of single-stranded, biotin-labeled oligonucleotide, 20 mM KCl, 2 mM MgCl₂, 4 mM EDTA, 1% glycerol, 1ng poly-dI-dC, and 0.025% NP-40 and binding buffer in a total volume of 20µL for 60 minutes at 37°C. To determine binding specificity, supershift analysis was performed with the addition of 0.5 µg of antibody to REF (Aviva Systems Biology, San Diego, CA) after 60 minutes of binding reaction and incubated for an additional 10 minutes at 37°C. Specific and nonspecific competitions were performed by using nonbiotinylated oligonucleotide of specific sequences and poly-dI-dC, respectively (data not shown). The samples were then run on a 6% DNA retardation gel (Invitrogen Inc.) at 100 V for 60 minutes. The gels were electrophoretically transferred at a constant current of 380 mA for 1 hour on ice to a positively charged nylon membrane (Pierce Biotechnology, Rockland, IL) and the membrane was immediately UV cross-linked for 60 seconds at 120 mJ/cm² (using a UV transilluminator equipped with 254-nm bulbs). Streptavidin
horseradish peroxidase conjugate and the light shift chemiluminescent substrate were used to
detect the biotin end-labeled DNA. Nylon membranes were then exposed to X-ray film for
detection.

**Filter Binding Assay.** Filter binding assays for relative quantification of protein-DNA
complexes were performed using grade 1 filters (Whatman, Florham Park, NJ) and cut precisely
in the shape of 8-mm discs by using a punch. The filters were then soaked in 0.5XTBE buffer for
at least 30 minutes, each filter disc was placed in an 8-mm column, and vacuum was applied
with a vacuum station (Qiagen, Valencia, CA). The vacuum was adjusted so that the filtering rate
was slow enough not to dry the filters. REF recombinant and deiminated (10 \( \mu \)g) was incubated
using the same EMSA condition described previously. In the control experiments, REF proteins
were omitted from the binding reactions. The reaction mix was then added to the column and
vacuum was applied. After the samples had passed through, the filters were washed once with
the same sample volume of 0.5X TBE and air dried. The filter discs were cross-linked at 120
\( mJ/cm^2 \) for 60 seconds in a UV transilluminator. The detection of biotin-labeled DNA by
chemiluminescence was performed according to the manufacturer’s instructions (cat. no. 20148;
Pierce Biotechnology). The PVDF membrane was developed using Fujifilm LAS-4000 machine.
The images obtained from the gel mobility shift assays and filter binding assays on films were
subjected to densitometric scan on a commercial imaging system (Alpha Innotech, San Leandro,
CA) and relative quantification were performed with the system-associated software (Alpha Ease
FC; Alpha Innotech). For relative quantification, all relative calculations were performed on the
same film and a relative ratio of total area was determined. For semi-quantitative estimates,
known band area values in the same film were used for comparison with unknowns. Some bound
filters were also subjected to luminometric counting on a scintillation counter. A linear
correlation was found between luminometric and film-based measurements performed for filter binding assays. All experimental results were subjected to statistical analyses where appropriate paired t-tests were performed.

**ATP synthase activity measurement.** The assay was performed with 10-15µg of mitochondrial protein at 37°C using the 0.12mM NADH as a donor with 9-14unit/ml pyruvate kinase/lactic dehydrogenase enzymes (Sigma Chemical Co.), phosphoenolpyruvic acid (Sigma Chemical Co.), and 2 µg/ml antimycin in 100 mM Hepes-KOH buffer containing 10mM MgSO₄, pH 8.0. The baseline was recorded at 340nm wavelength before adding 2.5mM ATP and then the reaction was recorded at same wavelength when it reached linear phase. The reaction was recorded again after inhibited by 5 µg/ml oligomycin (Sigma Chemical Co.). ATP synthase activity was calculated using Beer-Lambert law equation(Lemaire and Dujardin, 2008; Rimoldi et al., 1982).

**Electron Microscopy.** Optic nerve tissues dissected from mice were fixed in 4% paraformaldehyde, 0.1 % Glutaraldehyde in 0.1M phosphate buffer, pH 7.5. The optic nerves were then dehydrated in a series graded ethanol-water mixture: 30%, 50% and 70% and then straight into LR White™ embedding resin and subjected to polymerization overnight at 60°C. The embedded tissue was cut into 65nm sections onto nickel grids (200 mes). The immunogold stainning was performed following previously published methods with minor modification (Roth, 1984). Briefly, nickel grids mounted with sections were floated for 1hr on a drop of 50 mM Tris acetate, pH 7.2, 250 mM sucrose, with 0.5% BSA. The specimen grids were incubated in a solution of primary antibodies against REF (Abcam), diluted 1:200 in Tris-sucrose buffer as described above. Controls were tissues incubated in the same buffer without primary antibody. After 1 hour incubation at room temperature, the grids were rinsed with Tris-sucrose without
BSA for 9-12 drops, followed by incubation in goat anti-mouse-immunoglobulin G gold (10 nm, Amersham), diluted 1:40 in Tris-sucrose buffer for 1 hr at room temperature. The grids were washed and Tris-sucrose buffer and the distilled water. Sections were stained with uranyl acetate followed by lead citrate and examined with a Philips CM10 electron microscope. Magnifications for electron microscopy 21,000x.

**Supplementary References**


Supplemental Tables

Table S1. Fold increase in mRNA species bound with deiminated (d+) REF compared to non-deiminated (d-)

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* Based on Microarray Analyses (GEO accession number GSE 11843)
### Table S2. Primers for different PCR experiments

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*RT-PCR=Reverse transcription PCR  
**Q-PCR=Quantitative real time PCR

### Table S3. Probes for EMSA

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<td>Probe 3</td>
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<td>Probe 4</td>
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Supplementary Figure S1. REF in mitochondrial fraction and its localization on the mitochondrial surface. (A) Demonstration of REF in mitochondrial fraction. (a') The input cytosolic (cyt) and mitochondrial (mt) extracts from ND4 mouse brain separated on a 10% SDS-PAGE. (b') SDS-PAGE fractionated anti-REF IP products from inputs visualized using silver staining. Bottom panel shows REF detection using Western analysis. (B) Control IP with Aldehyde dehydrogenase 1 (ALDH1-IgG) antibody, bottom panels show Western blot detection with ALDH1 and REF antibody as indicated. (C) Detection of ATP5b, ATM1 and actin (control) as indicated in ND4 mouse cDNA derived from IP products of retinal cyt and mt fractions by Reverse transcription-PCR using appropriate primer pairs. Control represents RT-PCR from an IP performed with an antibody unrelated to any known mammalian protein. Localization of REF in primary hippocampal neuron culture was detected by (D) Representative merged image of Tomm20 and REF antibody. (E) Representative merged image of ATP5b and Tomm20. White boxes indicated co-localization. Z axis magnified views are indicated by yellow arrows. Bar= 10µm. (F, G) Electron microscopic image of CD1 and ND4 mice optic nerve as indicated with no primary antibody (negative control) incubated with 10nm gold particle coupled secondary anti-mouse antibody.
Supplementary Figure S2. Oxidation by mitochondria from CD1 (WT) and ND4 mice brain and spinal chord in presence of 0.1 mM ADP. Oxygen uptake was measured polarographically at 30°C and pH 7.4 in a 0.3 ml reaction system. The non-synaptic and synaptic mitochondrial respiration is depicted by dashed and sold lines respectively. (A) Representative respiration rate profiles in mitochondria isolated from CD1 and (B) from ND4 brains. (C) Representative respiration profiles in mitochondria isolated from CD1 and (D) from ND4 spinal cord. (E) Respiration rates in synaptic (S) mitochondria isolated from ND4 Spines (n= 5) and the CD1 (n= 6) groups. (F) Respiration rates in non-synaptic (NS) mitochondria isolated from ND4 Spines (n= 5) and the CD1 (n= 6) groups. Rate of oxygen consumption was measured in the presence and in absence of ADP. Results are mean ± SD from at least three independent measurements. All data were subjected to two-tailed unpaired t-test with respect to controls (*), *P≤0.05.
Supplementary Figure S3 Comparison mitochondrial complexes activities between control (CD1) and ND4 mice. (A) Activities of the different complexes in synaptic mitochondrial isolated from ND4 Brain (n= 5) and the controls (n= 6) groups. (B) Activities of the different complexes in non-synaptic mitochondria isolated from ND4 Brain and the controls as indicated. Activities are expressed as percent of control CD1 mice for each individual complexes respectively. Mean ± SD from at least three independent measurements has been shown.
Supplementary Figure S4. Comparison of neurites outgrow after PAD2 and PAD4 siRNA treatment and efficiency of transfections. Representative images showing detection of Tomm20 (Cy5) and deimination (FITC) on the PC12 cell after PADs siRNA transfection. (A) Representative merged images of PC 12 cells transfected with control siRNAs unrelated to any known mammalian sequence, (B) Representative merged images of PC 12 cells transfected with PAD2 and PAD4 siRNAs. Deimination levels are shown as (A’) control (transfected with control siRNA) PC 12 cells, (B’) PC 12 cells transfected with PADs and PAD4 siRNAs. Arrow indicates neurite like projections. Bar= 10µm. (C) Comparison of neurites length between control and PADs siRNA treated PC 12 cell as indicated. Neurites length of PC 12 cells were measured from 5 different slides (2 neurons of each) using LAS AF (Leica Inc.) software. Results have been expressed as mean ± SD from a sum total of 10 different neurites. Data was subjected to two-tailed unpaired t-test with respect to controls, *P≤0.05. (D) Quantification of ATP5b and GAPDH mRNA expression level after siRNA inhibition (Fig 3E) by ImageJ program. Mean ± SD have been shown, a two-tailed unpairs t-test was performed with respect to controls, *P≤0.05.
Supplementary Figure S5. ATP5b and ATM1 levels and ATPase activity in response to REF siRNA treatment. (A) RT-PCR analyses for ATP5b and ATM1 as indicated in (a’) mitochondria (mt) and (b’) cytosol (cyt) in control and REF siRNA treated cells as indicated. Hollow and solid bar represents ATP5b and ATM1 respectively. Mean densitometric data (with standard deviation; from equal area of 0.04 sq. mm in ImageJ software) of RT-PCR from control and cells subjected to REF siRNA treatment have been shown. (B) ATPase activity after REF siRNA transfection relative to the controls as indicated. Mean± SD from three independent experiments have been shown, *p ≤0.03. (C) Relative ATP5b product accumulation in rabbit reticulocyte coupled transcription and translation (TNT) assay with 10µg isolated mitochondria. ELISA results (background corrected) in presence of deiminated (d+) and non-deiminated (d-) REF and with no exogenous REF (no REF) as control has been shown. ELISA performed in presence of a control plasmid was taken as background. Mean± SD from three independent experiments have been shown, *p ≤0.03.