Supplementary Data

Supplementary Methods

Protein identification

Proteins were identified in the Proteomic Unit of Institut de Recerca Vall d'Hebron (Barcelona). Protein spots of interest were manually excised from the gel, and an In-gel trypsin digestion was performed. The silver-stained spots were pretreated, before the dehydration step, with a 1:1 mixture of 30 m*M* potassium ferrocyanide:100 m*M* sodium thiosulphate for 20 min and then washed with milli-Q water for 20 min to destain them. After the dehydration step, 10 μl of autolysis-stabilized trypsin (2.6 ng/μl; Promega) was added to each spot. The incubation was performed at 30°C for 18 h. Tryptic digests were purified using ZipTip microtiter plates (Millipore). Matrix-assisted lazer desorption ionization mass spectrometric analysis of tryptic peptides was performed on an Ultraflex TOF-TOF instrument (Bruker). Samples were prepared using α-cyano-4-hydroxy-cinnamic acid as a matrix on anchor chip targets (Bruker). Identification of the proteins was carried out by peptide mass fingerprint data and/or by TOF-TOF PSD. Database searches (Swissprot 2010_05, 16251 sequences) were performed using the Mascot algorithm (Matrix Science) (Supplementary Table S1).

NAD-NADH determination

NAD^+ and NADH were quantified by the NAD cycling assay as previously described with some modifications (3, 7). For NAD^+ determination, 10 mg of frozen tissue was homogenized with a motor-driven grinder in 200 μl of ice-cold HCl (0.05 *M*). The homogenate was centrifuged (16,000 *g*, 5 min, 4°C), and then the supernatant was collected. After the second centrifugation (16,000 *g*, 5 min, 4°C), the supernatant was maintained at 4°C for 30 min. For the NADH determination, 10 mg of frozen tissue was homogenized with a motor-driven grinder in 200 μl of ice-cold NaOH (0.02 *M*). The homogenate was centrifuged (16,000 *g*, 5 min, 4°C), and then the supernatant was collected. After the second centrifugation (16,000 *g*, 5 min, 4°C), the supernatant was incubated at 60°C for 30 min to eliminate NAD^+.

After neutralization of both extractions with 0.2 *M* potassium phosphate buffer pH 7.5 on ice, homogenates were centrifuged for 5 min at 16,000 *g* and the supernatants were collected for NAD^+ or NADH measurement. Fifteen microliters of supernatant containing NAD^+ or NADH extraction was added to 300 μl of cycling buffer (100 m*M* TrisHCl, 5 m*M* EDTA, 0.5 m*M* MTT, 1 m*M* PES, and 0.15 mg/ml ADH) and incubated for 5 min at 37°C. Then, 15 μl of EtOH (0.6 *M* final concentration) was added to start the reaction, and then the mixture was centrifuged for 30 s at 16,000 *g*. The absorbance of supernatant was measured at 570 nm after 10 min in a microplate spectrophotometer (PowerWave Microplate Spectrophotometer, BioTek). For quantification, values of the linear increase in absorbance were compared with values of NAD^+ or NADH standards. Data were normalized to milligram of tissue.

NADP-NADPH determination

$NADP^+$ and NADPH were quantified by the NADP cycling assay as previously described with some modifications (3, 7). For $NADP^+$ determination, 30 mg of frozen tissue was homogenized with a motor-driven grinder in 200 μl of ice-cold HCl (0.05M). The homogenate was centrifuged (16,000 *g*, 5 min, 4°C), and then the supernatant was collected. After a second centrifugation (16,000 *g*, 5 min, 4°C), the supernatant was maintained at 4°C for 30 min. For the NADPH determination, 30 mg of frozen tissue were homogenized with a motor-driven grinder in 200 μl of ice-cold NaOH (0.02M). The homogenate was centrifuged (16,000 *g*, 5 min, 4°C), and then the supernatant was collected. After a second centrifugation (16,000 *g*, 5 min, 4°C), the supernatant was incubated at 60°C for 30 min to eliminate $NADP^+$.

After neutralization of both extractions with 0.2 *M* potassium phosphate buffer pH 7.5 on ice, homogenates were centrifuged (16,000 *g*, 5 min, 4°C) and the supernatants were collected for $NADP^+$ or NADPH measurement. Thirteen microliters of supernatant containing $NADP^+$ or NADPH extraction were added to 300 μl of cycling buffer (100 m*M* TrisHCl, 5 m*M* EDTA, 0.5 m*M* MTT, 1 m*M* PES, 10 m*M* G6P, and 3 U/mL of G6PDH) and incubated for 5 min at 37°C. Then, 3.75 μl of SDS 1% were added to stop the reaction; the mixture was centrifuged (16,000 *g*, 1 min, RT). The absorbance was measured at 570 nm in a spectrophotometer (PowerWave Microplate Spectrophotometer, BioTek). For quantification, values of the linear increase in absorbance were compared with values of $NADP^+$ or NADPH standards. Data were normalized to milligram of protein.

Q-TOF based GSH analyses

Spinal cord samples were homogenate with a buffer containing 200 m*M* methane sulphonic acid with 5 m*M* DTPAC as previously described (2). Briefly, 200 μl of buffer were added to each sample, previously weighted, and were homogenated using an homogenator; and the protein content was analyzed using a NanoDrop at $\lambda = 280$ nm. Then, the samples were centrifugated for 30 min at 14000 *g* at 4°C and, finally, the supernatant were collected and filtrated in an eppendorf UltraFree 5 kDa filter. Four microliters of extracted sample was applied to a reverse-phase column (C18 Luna 3n pfp (2) 100A 150*2 mm, Phenomenex). The flow rate was 200 μl/min with solvent A (water) and solvent B (95% acetonitrile, 5% water containing corresponding 0.1% formic acid as counterion). The gradient consisted of a gradient of solvent B from 5% to 100% in 20 min, held at 100% solvent B for 5 min, and re-equilibrated at 5% solvent B for 6 min. Data were collected in positive electrospray mode in a QTOF (Agilent) operated in full-scan mode at 100–3000 m/z. The capillary voltage was 3500 V with a scan rate of 1 scan/s. N_2 was used as a gas nebulizer (flow was 5 l/min, and temperature was 350°C). We used the MassHunter Data Analysis Software (Agilent) to collect the results and the MassHunter Qualitative Analysis (Agilent) to perform the integration and metabolite quantitation. The identity of metabolite was confirmed by identity of mass, isotopic distribution, and coelution with authentic standards. The *m/z* values used for quantification were as follows: *m/z* 308.0919 $[M+H]^+$ for glutathione. Δ between calculated M.W. and detected masses was lower than 0.001 Da.

Respiratory chain activity

We quantified the activities of complex I plus complex III, complex II plus complex III, complex IV, and Citrate synthase in the spinal cord samples from 12 month-old *Abcd1*$^{-}$ mice as previously described (1, 4–6). The respiratory chain enzyme activities were determined in homogenates of whole spinal cords extracts in SETH buffer (sucrose 250 mmol/L, EDTA 2 mmol/L, Tris HCl 10 mmol/L, heparin 50 U/ml, and pH 7.4).

Complex I+III activity (EC 1.6.2.1). Enzymatic determination of NADH cytochrome c reductase rotenone sensitive is based on the reduction rate of cytochrome C by this enzyme in the presence of NADH and rotenone. It monitors the increase in absorbance at 550 nm in the presence and absence of rotenone. The rotenone-resistant activity was subtracted from the total activity of NADH cytochrome c reductase to obtain the cytochrome c reductase activity sensitive to rotenone (5). This determination is made in the presence of KCN (inhibitor of complex IV).

Complex II+III activity. Succinate cytochrome c oxidoreductase activity (EC 1.3.2.2, complexes II and II) is obtained by measuring the reduction of cytochrome c in the presence of succinate and the enzyme. The tissue homogenate is incubated with oxidized cytochrome c, the formation of reduced cytochrome c is monitored spectrophotometrically by measuring the increase in absorbance at 550 nm (1). This determination is made in the presence of rotenone and sodium azide (inhibitors of complexes I and IV, respectively).

Complex IV or cytochrome C oxidase activity (EC 1.9.3.1). Complex IV activity is measured using reduced cytochrome C as substrate. Cytochrome C oxidation is monitored at 550 nm (4).

Citrate synthase (EC 4.1.3.7) activity. Citrate synthase activity is used as a marker of mitochondrial content. Citrate synthase catalyzes the acetyl-coenzyme A oxalacetate reaction resulting citrate and Coenzyme A. The last product can be measured using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction is monitorized at 412 nm (6).

Supplementary References

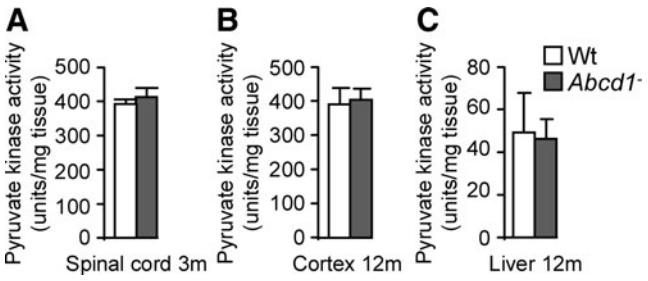
1. Fischer JC, Ruitenbeek W, Berden JA, Trijbels JM, Veerkamp JH, Stadhouders AM, Sengers RC, and Janssen AJ. Differential investigation of the capacity of succinate oxidation in human skeletal muscle. *Clin Chim Acta* 153: 23–36, 1985.
2. Lakritz J, Plopper CG, and Buckpitt AR. Validated high-performance liquid chromatography-electrochemical method for determination of glutathione and glutathione disulfide in small tissue samples. *Anal Biochem* 247: 63–68, 1997.
3. Requardt RP, Wilhelm F, Rillich J, Winkler U, and Hirrlinger J. The biphasic NAD(P)H fluorescence response of astrocytes to dopamine reflects the metabolic actions of oxidative phosphorylation and glycolysis. *J Neurochem* 115: 483–492, 2011.
4. Rustin P, Chretien D, Bourgeron T, Gerard B, Rotig A, Saudubray JM, and Munnich A. Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* 228: 35–51, 1994.
5. Sottocasa GL, Kuylenstierna B, Ernster L, and Bergstrand A. An electron-transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J Cell Biol* 32: 415–438, 1967.
6. Srere PA. An eclectic view of metabolic regulation: control of citrate synthase activity. *Adv Enzyme Regul* 9: 221–233, 1970.
7. Zerez CR, Lee SJ, and Tanaka KR. Spectrophotometric determination of oxidized and reduced pyridine nucleotides in erythrocytes using a single extraction procedure. *Anal Biochem* 164: 367–373, 1987.

Supplementary Table S1. List of the Oxidized Proteins Identified in 12 Month-Old *Abcd1*$^{-}$ Spinal Cord and in Human X-ALD Fibroblasts by Mass Spectrometry

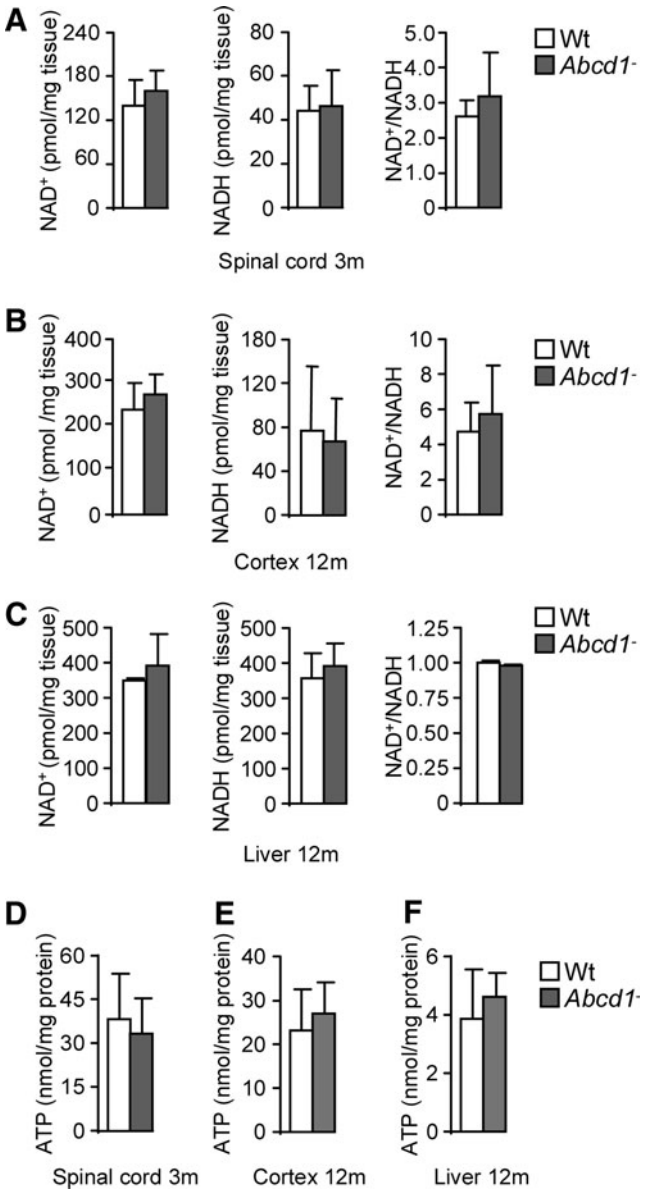
Spot no.	*Protein name*	*NCBI accession no.*	*Theoretical MW (kDa)*	*Theoretical pI*	*MOWSE score*	*Percent sequence coverage*	*No. of matching peptides*
1	Aldolase	GI:113607	39.35	8.30	77	58	21
2	Phosphoglycerate kinase	GI:146345481	44.55	8.02	70	43	13
3	Pyruvate kinase	GI:146345448	57.84	7.07	124	36	18
4	Dihydrolipoyl dehydrogenase	GI:118572640	54.75	7.99	90	19	8
5	Aconitase	GI:60391212	86.15	8.08	93	13	8
1	Pyruvate kinase	GI:17391514	58.47	7.96	132	36	10

Supplementary Table S2. List of Primers to Detect *Pkm2* Expression Levels

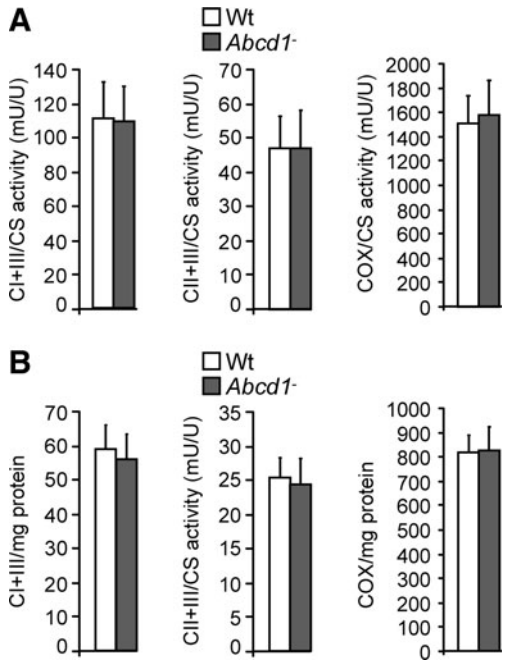
Gene	*Primer name*	*Sequence (5′–3′)*	*Product size*	*Tm (°C)*	*Elongation time (s)*
Pkm2	mPKM2_72-F	CTGTGTAAGGATGCCGTGCT	216	58	10
Pkm2	mPKM2_287-R	AGGGACAGGGGCTAGAAGAG	216	58	10
36b4	m36b4-F	ATGGGTACAAGCGCGTCCTG	73	58	10
36b4	m36b4-R	GCCTTGACCTTTTCAGTAAG	73	58	10



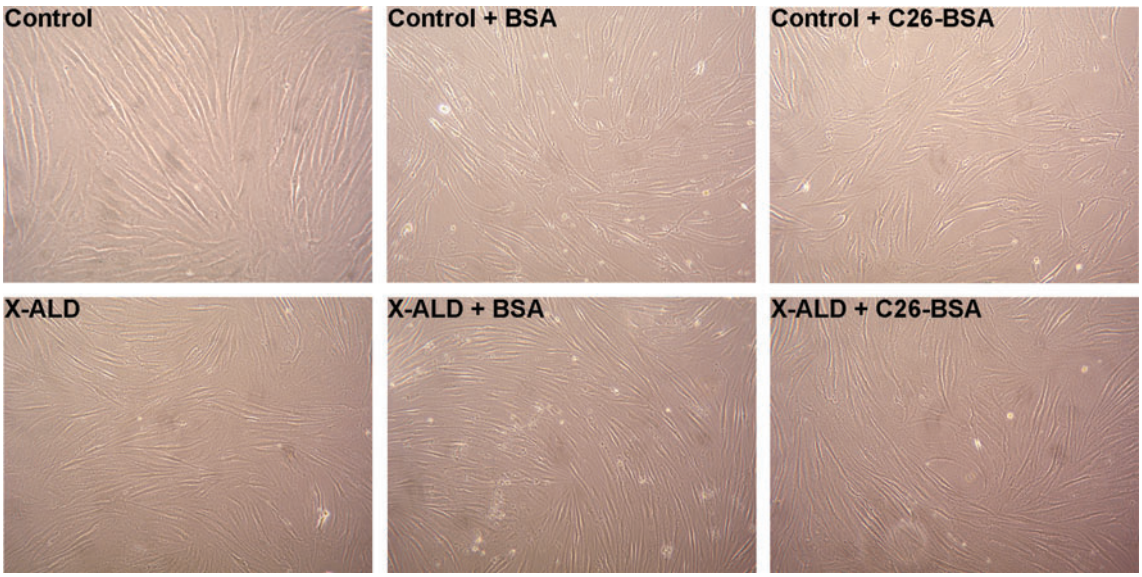
SUPPLEMENTARY FIG. S1. Pyruvate kinase activity was quantified in spinal cord from 3 month-old Wt and *Abcd1*⁻ mice (A), in cortex (B) and in liver (C) from 12 month-old Wt and *Abcd1*⁻ mice ($n = 6$/genotype). Statistical analysis was done with Student's *t*-test.



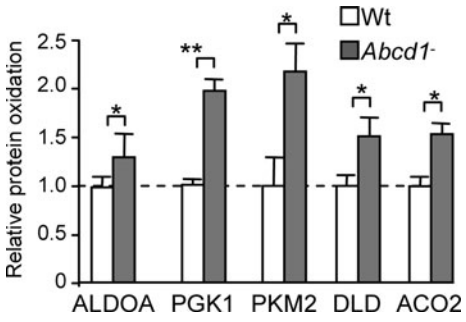
SUPPLEMENTARY FIG. S2. NADH, NAD^+ levels, and NAD^+/NADH ratio were quantified in spinal cord from 3 month-old Wt and *Abcd1*⁻ mice (A) and in cortex (B) and in liver (C) from 12 month-old Wt and *Abcd1*⁻ mice ($n = 6$/genotype); ATP levels were measured in spinal cord from 3 month-old Wt and *Abcd1*⁻ mice (D) and in cortex (E) and in liver (F) from 12 month-old Wt and *Abcd1*⁻ mice ($n = 6$/genotype). Statistical analysis was done with Student's *t*-test.



SUPPLEMENTARY FIG. S3. Mitochondria respiratory chain activities. NADH:cytochrome c oxidoreductase (CI + CIII), succinate:cytochrome c reductase (CII + CIII), and cytochrome c oxidase (COX) activities in spinal cord from 12-month-old Wt and *Abcd1*$^{-}$ mice. Activities are reported related to citrate synthase (mU/U CS) **(A)** and to the quantity of protein **(B)**.



SUPPLEMENTARY FIG. S4. Control and X-ALD fibroblasts cultured for 7 days in a medium containing 10% FCS, 0% FCS, and BSA or 0% FCS and C26-BSA.



SUPPLEMENTARY FIG. S5. Relative protein oxidation levels are expressed as a percentage of control. Statistical analysis was done by Student's *t*-test: $*p < 0.05$, $**p < 0.01$.