Modulation of mitochondrial and inflammatory homeostasis through RIP140 is neuroprotective in an adrenoleukodystrophy mouse model

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ABSTRACT

Aims
Mitochondrial dysfunction and inflammation are at the core of axonal degeneration in several multifactorial neurodegenerative diseases, including multiple sclerosis, Alzheimer's and Parkinson's disease. The transcriptional coregulator RIP140/NRIP1 (receptor-interacting protein 140) modulates these functions in liver and adipose tissue, but its role in the nervous system remains unexplored. Here, we investigated the impact of RIP140 in the Abcd1- mouse model of X-linked adrenoleukodystrophy (X-ALD), a genetic model of chronic axonopathy involving the convergence of redox imbalance, bioenergetic failure and chronic inflammation.

Methods and results
We provide evidence that RIP140 is modulated through a redox-dependent mechanism driven by very long-chain fatty acids (VLCFAs), the levels of which are increased in X-ALD. Genetic inactivation of RIP140 prevented mitochondrial depletion and dysfunction, bioenergetic failure, inflammatory dysregulation, axonal degeneration and associated locomotor disabilities in vivo in X-ALD mouse models.

Conclusions
Together, these findings show that aberrant overactivation of RIP140 promotes neurodegeneration in X-ALD, underscoring its potential as a therapeutic target for X-ALD and other neurodegenerative disorders that present with metabolic and inflammatory dyshomeostasis.
Introduction

Nuclear receptor-interacting protein 1 (NRIP1), also known as receptor-interacting protein 140 (RIP140), is a coregulator of nuclear receptors and other transcription factors. RIP140 plays a role in female fertility, obesity, insulin resistance, metabolic dysregulation, cancer, and inflammation [1, 2]. Remarkably, RIP140 can act as a coactivator and a corepressor, suggesting that it has a Janus-like dual function. Indeed, RIP140 negatively modulates energy homeostasis by regulating glucose and lipid metabolism in skeletal muscle, the liver, the heart, and adipose tissue through its interaction with PGC-1α. The importance of RIP140 in energy expenditure regulation has been demonstrated in RIP140-deficient mice, which show lower levels of stored triglycerides, greater resistance to diet-induced obesity, higher glucose clearance and insulin sensitivity, and a higher number of mitochondria in muscle and adipose tissue than control mice [3-7]. In addition, RIP140 positively regulates the inflammatory response in macrophages by interacting with NFKB (nuclear factor kappa B), which modulates Toll-like receptor-induced (TLR) pro-inflammatory cytokines such as TNFα and IL1β [8, 9]. Evidence for the role of RIP140 in the nervous system is scarce. The only reports available to date show that RIP140 content is increased in the hippocampus of individuals with Down syndrome (DS) [10] and decreased in Alzheimer’s disease (AD) patients [11] and that cognitive function is impaired in RIP140-deficient mice [12].

X-linked adrenoleukodystrophy (X-ALD, McKusick no. 300100) is the most common leukodystrophy with an incidence of 1:14,700 births [13]. This rare neurometabolic disease is caused by mutations in the ABCD1 gene, which encodes a peroxisomal transporter of very long-chain fatty acids (VLCFAs) [14]. Subsequently, VLCFAs accumulate in plasma and tissues such as the cerebral white matter, spinal cord, and adrenal cortex and constitute diagnostic biomarkers [15]. There are two main forms of the disease [16, 17]: i) childhood cerebral adrenoleukodystrophy (cALD) or cerebral adrenomyeloneuropathy (cAMN), a
rapidly progressing cerebral demyelinating leukodystrophy that leads to death (35-40% of cases), and ii) AMN, which encompasses 60% of cases, affects adult men and heterozygous women over the age of 40 [18] and is characterized by distal axonopathy involving the corticospinal tract in the spinal cord and peripheral neuropathy. AMN may switch to the cerebral inflammatory phenotype (cAMN) at any stage due to unknown triggers.

The precise role of VLCFAs in X-ALD pathogenesis and the factors that account for the diverse clinical phenotypes and prognosis remain elusive. Redox imbalance, energy metabolism failure, mitochondrial malfunction and inflammation are common features of most neurodegenerative disorders [19]. They also play an early and major role in the pathophysiology of X-ALD [20-26]. A mouse model of X-ALD (Abcd1- mice) and cerebral ALD patients share a similar transcriptional signature characterized by dysregulated mitochondrial biogenesis, a pathway controlled by the SIRT1/PGC-1A/PPARG axis [26, 27], and chronic inflammation, among other pathway alterations [28]. A low-grade pro-inflammatory status is also present in AMN patients and Abcd1- mice [29-31], suggesting that immune cells may also drive AMN. Indeed, the replacement of microglia and brain macrophages by CD34+ progenitors is necessary and sufficient to stop the progression of the cerebral forms of X-ALD in their early stages [32-36]. Microglial activation is a pathogenic event that precedes phagocytosis and synapse loss in X-ALD mice [37] and favours the opening of the blood-brain barrier [38]. Furthermore, VLCFAs accumulate in monocytes/macrophages but not T cells in AMN patients [39]. Upon myelin ingestion, the repolarization of macrophages from a pro-inflammatory to an anti-inflammatory state is not fully reversed in AMN. Enlarged lipid-laden macrophages positive for the pro-inflammatory marker CD86 are prominent in cALD lesions [31]. In addition, redox modulation of inflammation by VLCFAs has been observed in X-ALD. cALD monophagocytic cells secrete pro-inflammatory cytokines and exhibit lower intracellular glutathione levels in response to
VLCFA stimulation, which can be normalized by the addition of antioxidants [40].

Remarkably, our clinical trial in AMN patients showed that antioxidants normalized the redox status and the levels of several pro-inflammatory markers [41].

Here, given the role of RIP140 in macrophage metabolism and the NFKB-mediated pro-inflammatory response [8, 9], we investigated its contribution to the pathophysiology of X-ALD. We generated a RIP140 knockout (Rip140−/−) mouse and crossbred it with X-ALD mouse models (Abcd1−/− and Abcd1−/−/Abcd2−/− mice). Then, we evaluated the most salient hallmarks of the disease: i) mitochondrial biogenesis and function; ii) energy metabolism; iii) neuroinflammation; iv) axonal degeneration; and v) locomotor function. The results reveal RIP140 as a potential therapeutic target for brain disorders that share mitochondrial dysfunction and chronic inflammation as key pathogenetic features.
### Materials and Methods

#### Reagents and antibodies

All reagents, unless stated otherwise, were purchased from Sigma–Aldrich. Detailed information about the antibodies used in this study is summarized in Supplementary Table 1.

#### Human samples

Brain tissue samples from X-ALD patients and age-matched controls were obtained from the National Institutes of Health (NIH) NeuroBioBank. Frozen blocks of normal-appearing white matter (NAWM) were dissected from the frontal or parietal lobes of controls and cALD and cAMN patients. All children and adults with cerebral ALD had the more frequent parieto-occipital form of cerebral ALD. White matter sections from X-ALD patients and controls were stained with luxol fast blue (LFB) to detect demyelination, demyelination edges, and normal-looking areas. Brain tissue sections were processed when two to three adjacent sections showed no sign of demyelination by LFB staining and no perivascular cuffs of lymphocytes by haematoxylin and eosin staining. Detailed information about the brain samples, including the age of the patients and the postmortem interval until the sample was processed, is summarized in Supplementary Table 2.

#### Mouse experiments

All methods employed in this study were performed in accordance with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals (Guide, 8th edition, 2011, NIH) and European (2010/63/UE) and Spanish (RD 53/2013) legislation. Experimental protocols were approved by IDIBELL, the IACUC (Institutional Animal Care and Use Committee) and regional authority (3546 DMAH, Generalitat de Catalunya, Spain). The IDIBELL animal facility is accredited by The Association for Assessment and Accreditation
of Laboratory Animal Care (AAALAC, Unit 1155). Animals were housed at 22°C under specific-pathogen-free conditions on a 12-hour light/dark cycle and provided ad libitum access to food and water. Cages contained three to four animals. Histological and behavioural experiments were performed in a blinded manner with respect to genotype.

**Generation of RIP140 knockout mice with eGFP knock-in**

A conditional RIP140 gene knockout mouse with eGFP knock-in was generated by Ozgene on a pure C57BL/6J background (Fig. S2a). The complete details of the knockout mouse generation are located in **Supplementary Methods**. Mice heterozygous for the RIP140 floxed allele (conditional RIP140 mice) were crossed with mice expressing Cre recombinase under the control of the CMV promoter to generate RIP140 heterozygous (Rip140+/−) mice. We confirmed that the RIP140 protein was not expressed in Rip140−/− mouse tissues by western blotting using a RIP140 antibody (Fig. S2c).

**X-ALD mice**

We used two X-ALD mouse models in this study: Abcd1−/− and Abcd1−/−/Abcd2−/− mice. Abcd1 and Abcd2 gene inactivation has been described previously [42, 43]. These mice were crossbred with male Rip140−/− mice to obtain double knockout male Abcd1−/−/Rip140−/− and female Abcd1−/−/Rip140−/− mice as well as triple knockout male Abcd1−/−/Abcd2−/−/Rip140−/− and female Abcd1−/−/Abcd2−/−/Rip140−/− mice. Indeed, female Rip140−/− mice were infertile, as already described for another RIP140-deficient mouse [44]. To maintain the two colonies, male Abcd1−/−/Rip140−/− and female Abcd1−/−/Rip140−/− mice as well as male Abcd1−/−/Abcd2−/−/Rip140−/− and female Abcd1−/−/Abcd2−/−/Rip140−/− mice were crossbred. All mice used for the experiments were on a pure C57BL/6J background. All mice described developed to maturity without any abnormal clinical signs except infertility in female Rip140−/− mice [44].
We characterized the effect of RIP140 deletion on molecular defects observed in AMN patients in *Abcd1*/*Rip140*+/- mice at 12 months of age. *Abcd1* mice exhibit oxidative stress [22] and defects in energy metabolism [23] before AMN-like neuropathological signs emerge at 20 months of age [43].

To address the therapeutic effect of RIP140 ablation, we evaluated axonal degeneration and behavioural abnormalities in *Abcd1*/*Abcd2*+/-/*Rip140*+/- mice at 12 and 18 months of age. *Abcd2* is the closest homolog of *Abcd1*, and the metabolic functions of these genes overlap [45]; *Abcd2* has been postulated to act as a modifier of biochemical defects in X-ALD [46]. Compared to control mice, *Abcd1*/*Abcd2*+/- mice display increased VLCFA accumulation in the spinal cord [47], higher levels of oxidative damage to lipids and proteins [22], and a more severe AMN-like pathology with an earlier onset at 12 months of age [47], which makes them a more suitable model for therapeutic assays.

An antioxidant cocktail (N-acetyl-L-cysteine, alpha-lipoic acid and alpha-tocopherol) was administered as described previously [23]. To perform biochemical analysis, we euthanized the mice and quickly froze their tissues in liquid nitrogen and stored them at -80°C.

**High-resolution respirometry**

O₂ consumption in sets of five permeabilized lumbar spinal cord slices (LSCSs) (n=5 mice per genotype) was measured at 37°C in MiR05 medium, pH 7.4, by high-resolution respirometry using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) as previously described [25, 27].

**ATP measurement**
ATP content was measured by a chemiluminescence system using ATPlite 1step (PerkinElmer, Inc., Waltham, MA, USA) as previously described [23].

NAD⁺-NADH determination

NAD⁺ and NADH levels in the mouse spinal cord were quantified by the NAD cycling assay as previously described [23]. The data were normalized per milligram of tissue.

Quantitative real-time PCR

DNA extraction, RNA extraction, reverse transcription into cDNA, and quantitative RT-PCR analysis were performed as previously described [27], using standardized TaqMan® probes (Supplementary Table 3). To quantify mouse mtDNA content, we used primers for mouse cytochrome b (Cytb) and quantified mtDNA levels relative to nDNA levels that were determined by amplification of the intron-less mouse nuclear gene Cebpa [26]. Transcripts were quantified in triplicate for each sample.

Immunoblotting

Mouse and human tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer and immunoblotted using the antibodies described in Supplementary Table 1, as previously described [30]. The protein bands were quantified by densitometry using ImageJ v1.50i (NIH, Bethesda, MD, USA).

Immunohistochemistry/Immunofluorescence

IHC studies performed in WT, Abcd1+/Abcd2−/−, Abcd1+/Abcd2−/−/Rip140−/− and Rip140−/− mice were carried out by the avidin-biotin peroxidase method, as reported previously [27]. The number of GFAP⁺ cells (astrocytes) and Iba1⁺ positive cells (microglia) per mm² in the spinal
cord ventral horn were determined by counting brown-coloured cells with the Cell Counter plugin in ImageJ [30]. The data are presented as the average number of cells in two 20x images per animal from each group. The researcher was blinded to genotype when analysing the images.

For GFP immunofluorescence (IF) studies, the spinal cords and brains of one-month-old WT and Rip140$^{-/-}$ mice were equilibrated in 30% sucrose after the mice were perfused. Tissues were embedded in tissue freezing medium (Sakura) inside disposable plastic moulds (Sakura). Serial sections (5 µm thick) were cut along the coronal and sagittal planes with a cryostat (1950M; Leica Biosystems). IF was performed as described previously [48] using the antibodies indicated in Supplementary Table 1. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Thermo Fisher Inc.). Confocal images were taken using the 40x objective of a Leica TCS SP5 confocal microscope (Leica Biosystems). Sections from WT mice were used to measure the specific eGFP signal in sections from Rip140$^{-/-}$ mice.

Organotypic spinal cord slice cultures

Organotypic spinal cord slice cultures (OSCSC) were prepared as previously described [22]. The slices were incubated at 37°C, and the medium was changed twice a week. After two weeks of culture, the OSCSCs were treated with C26:0 (100 µM, dissolved in ethanol), H$_2$O$_2$ (10 µM) and/or Trolox (500 µM) [22].

Behavioural experiments

The same mice were subjected to the treadmill and bar cross tests at 12 and 18 months of age as previously described [27, 47, 49]. One Abcd1$^{-/-}$Abcd2$^{-/-}$ mouse died between the ages of 12 and 18 months. Hind limb clasping was assessed by suspending WT, Abcd1$^{-/-}$Abcd2$^{-/-}$, Abcd1$^{-/-}$
/Abcd2<sup>−<sub>−</sub></sup>/Rip140<sup>−<sub>−</sub></sup> and Rip140<sup>−<sub>−</sub></sup> mice from their tails until they reached a vertical position. The hind limb extension reflex was analysed over a period of 10 s in three consecutive trials separated by 5 minutes of rest. The hind limb extension reflex was scored as described in Supplementary Table 4, as adapted from Dumser et al. [50]. The researcher was blinded to the genotype of the mice while performing these behavioural tests.

**Statistical analysis**

The values are presented as the means ± standard deviations (SDs) or, when explicitly noted, as the standard errors of the mean (SEMs). Significant differences (*<sup>p</sup><0.05, **<sup>p</sup><0.01, ***<sup>p</sup><0.001) between two groups were determined by two-tailed unpaired Student’s t-test. When comparing more than two groups, significant differences were determined by one-way or two-way ANOVA followed by Tukey’s *post hoc* test or the Kruskal-Wallis non-parametric test followed by Dunn’s *post hoc* test after verifying normality (Shapiro-Wilk’s test). Statistical analysis was performed using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla, California, USA). Exact p-values are reported in Supplementary Table 5.
Results

RIP140 expression is elevated in the spinal cords of Abcd1− mice and the white matter of cALD patients

The X-ALD mouse model and human patients share an abnormal metabolic gene expression signature characterized by mitochondrial DNA (mtDNA) depletion and dysfunction, an NFKB-mediated pro-inflammatory response, and other components [28]. We observed increases in both RIP140 mRNA and protein levels in the spinal cords of Abcd1− mice at the age of 12 months, (Fig. 1a and 1b), which is 6 months before disease onset. Dysregulation of RIP140 protein levels was organ-specific, as we did not observe any change in non-affected tissues in this mouse model, such as the cerebral cortex and liver (Fig. S1a).

Next, we analysed whether redox status regulates RIP140 expression, given the decisive role of oxidative stress in the pathogenic cascade of X-ALD pathophysiology [22]. First, we observed that RIP140 protein levels were normalized in the spinal cords of 12-month-old Abcd1− mice spinal cord after administration of an antioxidant cocktail composed of N-acetyl-L-cysteine, alpha-lipoic acid, and alpha-tocopherol [23] (Fig. 1c), indicating that an increase in RIP140 levels may be redox-dependent. To explore the molecular mechanism underlying this phenomenon, we used ex vivo organotypic spinal cord slice cultures (OSCSCs) from WT and Abcd1− mice as previously described [22]. We first verified that Rip140 mRNA expression was increased in Abcd1− OSCSCs compared with WT OSCSCs at baseline (Fig. S1b). Next, we revealed that upon the addition of H₂O₂ or C26:0 (the VLCFA that accumulates in X-ALD) to the OSCSC culture medium, expression of RIP140 (both protein and mRNA) is induced in a redox-dependent manner (Fig. 1d-e and Fig. S1b-d). We confirmed that the induction of RIP140 involved a redox-dependent mechanism since the
addition of Trolox (500 µM), an analogue of α-tocopherol, prevented the C26:0-mediated increase in RIP140 expression (Fig. 1e and Fig. S1c-d).

We also observed increased expression of RIP140 in the normal-appearing white matter (NAWM) of cALD patients, but not in the NAWM of cAMN patients, compared with the NAWM of age- and sex-matched control subjects (Fig. 1f and g).

**RIP140 localization in the adult mouse central nervous system**

The abovementioned results demonstrate that RIP140 is induced in Abcd1− mice, apparently due to redox imbalance. To investigate the pathogenetic implications of RIP140 induction, we generated a germline RIP140 knockout (Rip140−/−) mouse line and crossbred it with X-ALD mouse models (Abcd1− and Abcd1−/Abcd2−/− mice). Since the coding sequence of RIP140 has been replaced by the eGFP gene in the KO animals, eGFP transcription is driven by the RIP140 promoter and thus reflects the expression of the endogenous RIP140 gene in wild-type (WT) mice (Fig. S2a). We then studied RIP140 protein localization in the mouse central nervous system by immunofluorescence using an anti-GFP antibody. Costaining of the spinal cord with the markers NeuN, Iba1, and Olig2 revealed that RIP140 was present in neurons (Fig. 2a-d), microglial cells (Fig. 2e-h) and Olig2+ oligodendrocytes (Fig. 2i-l). However, we did not detect RIP140 expression in GFAP+ astrocytes in the spinal cord or other areas of the central nervous system (Fig. S3a-d). We observed a similar pattern of RIP140 expression in other regions of the mouse central nervous system. RIP140 was present in neurons (Fig. S3e-g), microglia (Fig. S3h-j), and Olig2+ cells (Fig. S3k) in areas such as the cerebral cortex, cerebellum, hippocampus, and pons. RIP140 was present only in certain neuronal subpopulations in the cerebral cortex, as not all the NeuN+ cells were GFP+ (Fig. S3e). Thus, RIP140 is expressed in spinal cord motor neurons and microglia, cell types that show
neuropathological changes in Abcd1− mice, supporting the investigation of this coreceptor’s role in X-ALD.

**RIP140 deficiency prevents mitochondrial depletion and bioenergetic failure in X-ALD mice**

Since RIP140 is a master regulator of mitochondrial biogenesis, we sought to determine the effect of RIP140 deletion on mitochondrial dysfunction in the spinal cords of Abcd1− mice at 12 months of age [6, 7]. We observed that Rip140− mice exhibited increased mtDNA levels in the spinal cord, brown adipose tissue, and gastrocnemius and soleus muscles but not in the cerebral cortex, cerebellum, pons, hippocampus, white adipose tissue, liver, or heart at 3 months of age (Fig. S4). The most prominent increase in mtDNA was found in the gastrocnemius muscle. Loss of RIP140 on the genetic background of Abcd1− mice preserved mitochondrial DNA (mtDNA) levels (Fig. 3a) and the mRNA expression of mitochondrial biogenesis-associated genes in the spinal cord (sirtuin 1, Sirt1; peroxisome proliferator-activated receptor, gamma, coactivator 1-alpha, Ppargc1a; peroxisome proliferator-activated receptor gamma, Pparg; transcription factor A, mitochondrial, Tfam; and nuclear respiratory factor 1, Nrf1) (Fig. 3b). The modest decrease of the mitochondrial biogenesis factors and the mtDNA copy numbers in Abcd1− mice is consistent with a mild, chronic progressive axonopathy, and has been shown repeatedly and robustly reduced in the Abcd1− mice in all cohorts tested over a 10 years span [21, 26, 27, 30]. A lack of RIP140 also preserved the protein levels of NDUFB8 [NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8] and SDHB [succinate dehydrogenase complex, subunit B, iron sulfur (Ip)], which are complex I and complex II subunits, respectively, in the spinal cords of Abcd1+/Rip140− mice (Fig. 3c). These subunits have been repeatedly shown to be depleted in Abcd1− mice [26]
We assessed mitochondrial oxygen consumption \textit{ex vivo} in mouse spinal cord samples by respirometry (Oroboros). Importantly, loss of RIP140 increased oxygen consumption after the addition of ADP, ADP + succinate, or ADP + succinate + rotenone (State 3) and restored the respiratory control ratio (RCR) in the spinal cords of $Abcd1^-\text{/}Rip140^-$ mice (Fig. 3d), thus preventing defective mitochondrial respiration in the main target organ of the disease, as previously reported with pioglitazone treatment or Sirt1 activation strategies [25, 27].

We next analysed the effect of RIP140 genetic deletion on bioenergetics by measuring ATP, NAD$^+$, and NADH levels. A lack of RIP140 increased ATP (Fig. 3e) and NADH content (Fig. 3f), while NAD$^+$ levels were not altered in the spinal cords of 12-month-old $Abcd1^-\text{/}Rip140^-$ mice compared to those of 12-month-old $Abcd1^-$ mice (Fig. 3g).

On the other hand, we did not find defects in mitochondrial function, biogenesis or energy metabolism in 12-month-old $Rip140^-$ mice (Fig. 3a-g), which suggests that RIP140 is not essential for mitochondrial and metabolic housekeeping functions in the adult mouse spinal cord. Together, these findings illustrate that loss of RIP140 prevents mitochondrial depletion and dysfunction, as well as the associated bioenergetic deficits that are observed in the spinal cords of $Abcd1^-$ mice.

A lack of RIP140 prevents NFkB activation and dysregulation of the inflammatory profile in the spinal cords of $Abcd1^-$ mice

$Abcd1^-$ mice and AMN patients present with low-grade inflammatory dysregulation associated with NFkB pathway activation in the CNS [28, 29, 31]. RIP140 is required for the NFkB-mediated pro-inflammatory response [8, 9]. To ascertain whether RIP140 induction contributes to neuroinflammation in the context of X-ALD, we first examined the protein levels of the main components of the NFkB pathway after RIP140 genetic deletion. We reported activation of p65 and p52, components of the canonical and noncanonical NFkB...
pathways, respectively, and their regulators pIKBA and IKKB in the spinal cords of 12-month-old *Abcd1* mice (Fig. 4a and Fig. S5), which is consistent with previous findings [28]. Loss of RIP140 in *Abcd1* mice impeded NFKB pathway activation by reducing p65, p52, pIKBA and IKKB protein levels (Fig. 4a), whereas *Rip140*/* mice did not show activation of any NFKB-related proteins.

We next quantified the mRNA expression of several cytokines, chemokines and related inflammatory genes that are dysregulated in the spinal cords of 12-month-old *Abcd1* mice [30]. We revealed that RIP140 loss reduced the expression of several pro-inflammatory genes in the spinal cords of *Abcd1*/Rip140/* mice: chemokine (C-C motif) ligand 5 (*Ccl5*), chemokine (C-C motif) receptor type 6 (*Ccr6*), chemokine (C-X-C motif) ligand 9 (*Cxcl9*), chemokine (C-X-C motif) ligand 10 (*Cxcl10*), nuclear factor kappa B subunit 2 (*Nfkb2*), tumour necrosis factor alpha (*Tnfa*), and tumour necrosis factor receptor superfamily member 1a (*Tnfrsf1a*) (Fig. 4b). In addition, RIP140 deletion on the *Abcd1* genetic background increased the mRNA expression of some anti-inflammatory genes, such as resistin-like alpha (*Retnla*, also called *Fizz1*), insulin-like growth factor 1 (*Igfl*) and transforming growth factor beta 1 (*Tgfb1*) (Fig. 4c). No neuroinflammatory activation of NFKB or the tested cytokines was observed in *Rip140*/* mice. These data collectively provide evidence of the essential role of RIP140 in NFKB pathway activation and inflammatory profile dysregulation in the spinal cords of *Abcd1* mice.

**RIP140 deficiency halts axonal degeneration and neurological symptoms in *Abcd1*/Abcd2/* mice**

To evaluate the preclinical therapeutic potential of RIP140 inhibition, we assessed the impact of RIP140 loss on axonal degeneration and the locomotor phenotypes of *Abcd1*/Abcd2/* mice
We also evaluated these parameters in Rip140−/− mice to discard any harmful consequences of RIP140 deletion.

First, we examined the impact of RIP140 loss on the immunohistochemical signs of X-ALD neuropathology present in Abcd1−/−Abcd2−/− mice at 18 months of age. The following immunohistochemical parameters were assessed: (i) microgliosis detected with an Iba1 antibody; (ii) astrocytosis detected with a GFAP antibody; (iii) axonal degeneration determined by the accumulation of synaptophysin and amyloid precursor protein (APP) in axons; (iv) lipidic myelin debris detected by Sudan Black staining [47]; (v) oxidative damage to lipids and proteins indicated by malondialdehyde (MDA) staining [24]; (vi) damage to motor neurons reflected by reduced staining with an SMI-32 antibody, which labels a nonphosphorylated epitope of neurofilament proteins; and (vii) mitochondrial depletion indicated by decreased cytochrome c (Cyt C) staining in motor neurons [26] (Fig. 5a-g').

Loss of RIP140 in Abcd1−/−Abcd2−/− mice prevented microgliosis and astrocytosis, as reflected by reductions in the densities of Iba1+ and GFAP+ cells (Fig. 5a-h, g'). Loss of RIP140 blocked the accumulation of APP and synaptophysin in axons (Fig. 5i-p, g') and the appearance of myelin debris along the spinal cord (Fig. 5q-t). We also observed attenuation of oxidative damage (Fig. 5u-x), improvements in motor neuron health, and increased mitochondrial content (Fig. 5y-f'). These parameters were not different between Rip140−/− mice compared with WT mice (Fig. 5a-g'). In conclusion, loss of RIP140 conferred neuroprotection to the X-ALD mouse model.

Next, we assessed the impact of RIP140 deficiency on the locomotor phenotype of X-ALD mice at 12 and 18 months of age, time points at which neurological symptoms emerge and progress to a more advanced stage of neuropathology. The behavioural abnormalities of X-ALD mice were previously characterized by the bar cross, treadmill and clasping tests [24,
We found that loss of RIP140 in Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup> mice prevented hind limb clasp abnormalities (Fig. 6a-b) and locomotor impairment, as measured by the bar cross and treadmill tests, at 12 and 18 months of age (Fig. 6c-f). Loss of RIP140 did not have any harmful effect on the locomotor performance of Rip140<sup>−/−</sup> mice (Fig. 6a-f).

Overall, the absence of RIP140 prevented axonal degeneration and halted the onset of neurological symptoms in Abcd1<sup>−/−</sup>/Abcd2<sup>−/−</sup> mice.
Discussion

This work shows that genetic deletion of RIP140 is sufficient to prevent disease-associated mitochondrial dysfunction, bioenergetic deficits, the NFκB-dependent pro-inflammatory response, and oxidative damage thus providing functional neuroprotection in a preclinical X-ALD mouse model. The positive findings of this study warrant further testing of the role of RIP140 in other neurodegenerative diseases with similar pathophysiological features. Indeed, mitochondrial dysfunction, redox imbalance, and neuroinflammation are shared features of most multifactorial neurodegenerative diseases and X-ALD [52].

The role of RIP140 in neurodegenerative disorders is not well understood. Of note, RIP140 degradation is associated with the activation of stress responses in neurons [53], which suggests a neuroprotective effect of RIP140 inhibition in a stressful context. Moreover, RIP140 content is increased in the hippocampus of Down syndrome (DS) patients [10]. While this phenomenon is likely related to the location of the human NRIP1 gene on chromosome 21, inhibition of RIP140 is sufficient to restore mitochondrial function, intramitochondrial superoxide levels and bioenergetic deficits in human DS foetal fibroblasts [54]. Similar results were found in another study that used mice that were either trisomic (Ts3Yah) or monosomic (Ms3Yah) for a 9.2 Mb fragment on mouse chromosome 16 (syntenic to human chromosome 21) that includes Rip140 [55]. In addition, Ms3Yah mice produce less mitochondrial reactive oxygen species (ROS) than WT mice. Together, these results suggest that RIP140 plays a major role in mitochondrial function and ROS production in DS.

One of the main findings of this study is that RIP140 deletion has an anti-inflammatory effect in nervous tissue of the X-ALD mouse model. RIP140 is a coactivator of the NFκB-dependent pro-inflammatory response [8, 9], and responsible for the suppression of anti-inflammatory M2 macrophage polarization [56], which is recognized as a key factor...
in neurodegeneration and demyelination [57]. Here, loss of RIP140 led to normalization of
the inflammatory profile and prevented NFKB pathway induction in the spinal cords of X-
ALD mice. Moreover, we detected RIP140 expression in microglia, one of the primary cell
types implicated in neuroinflammation. Since we showed that RIP140 deficiency prevents
microgliosis in X-ALD mice, an exciting possibility worth investigating in future studies is
whether specific RIP140 repression in microglia/macrophages can reverse X-ALD pathology.

The redox-inflammatory interplay governed by RIP140 function is intriguing. The
central role of the reciprocal interplay between oxidative stress and inflammation is, in a
general notion, a hallmark feature in the genesis of diseases, ranging from genetically
determined disorders, such as Rett, Down syndrome, to the most common metabolic
dysfunctions, including diabetes, cardiovascular disease and neurodegenerative disorders,
such as AD and Parkinson’s disease [58-60]. In the X-ALD context, we formerly showed that
a chronic-low grade proinflammatory status exists in mouse and humans [28, 29, 41], and
that excess of the saturated fatty acid C26:0 disrupts the mitochondrial OXPHOS and induces
the generation of free radicals at the ETC from both complexes I and II, at least in human
fibroblasts [25]. In this study, we reveal that C26:0 excess induces RIP140 in a redox-
dependent manner. Indeed, H$_2$O$_2$ and, more importantly, C26:0 increased RIP140 expression
in OSCSCs from WT and Abcd1$^{-}$ mice. This phenomenon was reversed by the addition of
Trolox ex vivo or a cocktail of antioxidants in vivo, confirming the role of C26:0-mediated
oxidative stress in the induction of RIP140. This observation is in line with several reports
showing that excess of palmitic acid (C16:0), another saturated fatty acid producing
mitochondrial ROS [61, 62], also increases RIP140 expression, while stimulating the
secretion of inflammatory cytokines by peripheral blood mononuclear cells (PBMCs) [63] or
RAW264.7 macrophages [64]. RIP140 ignites inflammatory responses activating NFKB-
dependent cascades, including TNF-$\alpha$. Of note, TNF-$\alpha$ has been shown to induce
mitochondrial ROS production [65]; Kastl L et al. reported that TNF-α induced mitochondrial ROS through mitochondrial complex I, II and/or complex III, mimicking a mild uncoupling effect in murine hepatocytes [66], endothelial cells [67] or fibroblasts[68]. In light of these reports and our findings, we propose a “devil circle” type of situation operating in X-ALD: i) in first instance, fatty-acid mediated mitochondrial ROS induces RIP140; ii) in its turn, RIP140 activates NFKB and TNF-α (or other cytokines noxious for mitochondria); iii) more ROS are produced from mitochondria, which aggravates oxidative stress, thus inducing RIP140 further; iv) suppression of RIP140 leads to normalization of inflammatory cascades, mitochondria function and redox homeostasis, the later proven by normalized lipoxidative damage in neurons, visualized by MDA staining (see Fig. 7).

This model may be extrapolated to other neurodegenerative and metabolic diseases, in particular the inflammatory processes induced by excess saturated fatty acids, such as obesity and type 2 diabetes. Indeed, RIP140 is expressed at higher levels in adipose tissue macrophages of high-fat diet (HFD)-induced obese mice than those of normal diet-fed mice. Silencing of RIP140 expression in monocytes/macrophages improves HFD-induced insulin resistance, which is correlated with M2 macrophage polarization in adipose tissue [69]. RIP140 levels are also associated with the elevation of free fatty acids and pro-inflammatory cytokines in PBMCs from Type 2 Diabetes patients [63].

Another interesting aspect is that RIP140 mRNA and protein levels were elevated in the normal-appearing white matter (NAWM) of cALD patients, but not in that of cAMN patients, compared with the NAWM of healthy subjects. The preferential induction of RIP140 in cALD patients but not in cAMN patients suggests that RIP140 could be a differential biomarker of these two X-ALD phenotypes and deserves validation in independent cohorts. Most of the studies designed to identify biomarkers of the distinct clinical phenotypes of X-ALD were not conclusive [70]. Hence, RIP140 emerges as a
candidate driver of the inflammatory phenotype in the proposed “three-hit” hypothesis of the
disease pathogenesis of cALD [71]. We have previously reported different treatments that
have worked successfully in X-ALD mice, all of them based on modulating redox and
inflammatory therapeutic targets, shown dysregulated after applying multiomics approaches
[28, 29]. These therapeutic strategies include a combination of high-dose antioxidants [24],
the PPARγ agonist pioglitazone [26], SIRT1 activators like resveratrol [27], the UPR
inhibitor TUDCA [49], the NRF2 activator dimethyl fumarate [30], and lately high-dose
biotin (MD1003) [21]. Some of them have been tested in clinical trials, such as MD1003, a
natural derivative of pioglitazone, or the combination of antioxidants, the latter with
encouraging results [41]. All these dysregulated nodes converge in metabolic and
inflammatory pathways that are modulated by RIP140, which places this transcriptional co-
regulator at a centre stage in the pathophysiology of X-ALD.

In conclusion, we propose that RIP140 orchestrates fatty acid overload-mediated
oxidative stress, mitochondrial dysfunction, and neuroinflammation in X-ALD. We report
that RIP140 expression is induced in a mouse model of X-ALD and cALD patients and that
RIP140 deficiency exerts a neuroprotective effect in a mouse model of X-ALD. The results
of this study suggest that RIP140 inhibition may be an attractive novel strategy for halting
neurodegeneration in X-ALD and related disorders with shared features of redox imbalance,
mitochondrial damage, and neuroinflammation. Given that the inactivation of RIP140 from
conception does not significantly impact development, health, or life span in the mouse, with
exception on female fertility, we believe that significant redundancy in the modulation of
central metabolic and inflammatory pathways may be at play and imply that caution should
be exerted when translating these results to humans.
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Ethical approval

Brain tissue samples from X-ALD patients and age-matched controls were obtained from the National Institutes of Health (NIH) NeuroBioBank. All methods employed in this study were performed in accordance with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals (Guide, 8th edition, 2011, NIH) and European (2010/63/UE) and Spanish (RD 53/2013) legislation. Experimental protocols were approved by IDIBELL, the
IACUC (Institutional Animal Care and Use Committee) and regional authority (DMAH, Generalitat de Catalunya, Spain). The IDIBELL animal facility is accredited by The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC, Unit 1155).

Data Availability
This study includes no data deposited in external repositories. The data that support the findings of this study are available from the corresponding author upon request.
Author contributions

PRR, MFB, SF, and AP conceived the study. PRR, JG, LE, MR, NYC, and AN performed the experiments. PRR, JG, LE, A, MR, NYC, FV, AN, RP, IF, MPO, MFB, SF, and AP designed and/or interpreted aspects of the different experiments. AP administered the project and acquired funding. PRR, SF, and AP wrote the original draft. PRR, SF, and AP reviewed and edited the manuscript. All the coauthors provided input to the manuscript.

Conflict of interest

The authors declare that this article was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.
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Figure Legends

**Figure 1** RIP140 induction in the spinal cord of the X-ALD mouse model (Abcd1<sup>-</sup> mice) and the white matter of cALD patients

a) *Rip140* mRNA expression was measured in the spinal cords of WT (n=8) and *Abcd1<sup>-</sup>* (n=8) mice at 12 months of age. Gene expression was normalized to *Rplp0* expression and is presented as the fold change compared to the level in WT mice. b) Representative immunoblots (top) showing RIP140 protein expression in the spinal cords of WT (n=8) and *Abcd1<sup>-</sup>* (n=8) mice at 12 months of age. Protein content was normalized to γ-TUB expression and is presented as the fold change compared to the level in WT mice (bottom). c) Representative immunoblots (top) showing RIP140 protein expression in the spinal cords of WT (n=6), *Abcd1<sup>-</sup>* (n=6) and *Abcd1<sup>-</sup>* mice treated with an antioxidant cocktail for 4 months (*Abcd1<sup>-</sup> + Antiox.; n=6) at 12 months of age. Protein content was normalized to γ-TUB expression and is presented as the fold change compared to the level in WT mice (bottom). d) Representative immunoblots (top) showing RIP140 protein expression in OSCSCs from WT and *Abcd1<sup>-</sup>* mice treated with H<sub>2</sub>O<sub>2</sub> (10 µM) or vehicle (n=4 per genotype and treatment). The protein level was normalized to γ-TUB expression and is presented as the fold change relative to the level in vehicle-treated OSCSCs (bottom). e) Representative immunoblots (top) showing RIP140 protein expression in WT and *Abcd1<sup>-</sup>* OSCSCs treated with C26:0 (100 µM) and/or Trolox (500 µM) compared with vehicle-treated OSCSCs (WT n=5 per condition; *Abcd1<sup>-</sup>* n=4 per condition). The protein level was normalized to γ-TUB expression and is presented the fold change relative to the level in vehicle-treated OSCSCs (bottom). f) *RIP140* mRNA expression was measured in the cerebral normal-appearing white matter (NAWN) of control adults (n=23), control children (n=6), cAMN patients (n=17) and cALD patients (n=6). Gene expression was normalized to *RPLP0* expression and is presented as the fold change compared to the level in WT controls.
change relative to the level in control (CTL) patients (adults for cAMN and children for cALD). g) Representative immunoblots (left) showing RIP140 protein expression in the cerebral white matter of CTL subjects (n=6 adults and n=6 children) and cerebral NAWM of cAMN (n=6) and cALD (n=6) patients. Protein expression was normalized to γ-TUB expression and is presented as the fold change relative to the level in CTL patients (adults for cAMN and children for cALD) (right). The values are presented as the means ± SDs (a-e) or means ± SEMs (f-g). * p<0.05; ** p<0.01; *** p<0.001; (a, b, d) unpaired Student’s t-test or (e, f, g) one-way ANOVA or (e) two-way ANOVA followed by Tukey’s post hoc test.

Figure 2 RIP140 localization in neurons, microglia and Olig2+ cells in the spinal cords of adult mice

Immunofluorescence study of RIP140 localization, as determined by coimmunostaining for GFP and different cell type markers, in the spinal cords of 1-month-old Rip140−/− mice. Representative images from left to right: (a, e, i) green-labelled GFP-positive cells (GFP antibody); red-labelled (b) neurons (NeuN), f) microglial cells (Iba1) and j) Olig2+ cells (Olig2); (c, g, k) blue-labelled cell nuclei (DAPI); and (d, h, l) merged green (GFP) and red (cell type marker) images. The white arrows indicate cells double positive for GFP (green) and the corresponding cell type marker (red). The insets show green, red and merged signals at 2.5x (d and h) or 4.75x magnification (l). Scale bar = 100 µm.

Figure 3 RIP140 deletion preserves mitochondrial content and function and bioenergetics in the spinal cords of Abcd1− mice

a) mtDNA content in the spinal cords of WT (n=8), Abcd1− (n=8), Abcd1−/Rip140−− (n=8) and Rip140−− (n=8) mice at 12 months of age. mtDNA content is expressed as the ratio of mtDNA (Cytb) to nuclear DNA (nDNA, Cebpa) and is presented as the fold change compared to the
level in WT mice. **Sirt1, Pgc-1a, Pparg, Tfam and Nrf1** expression was measured in the spinal cords of WT (n=8), *Abcd1* (n=8) *Abcd1*/*Rip140−/− (n=8) and *Rip140−/− (n=8) mice at 12 months of age. Gene expression was normalized to *Rplp0* expression and is presented as the fold change compared to the level in WT mice. **Representative immunoblots (left) showing mitochondrial complex I (NDUFB8) and complex II (SDHB) protein expression in the spinal cords of WT (n=6), *Abcd1* (n=6), *Abcd1*/*Rip140−/− (n=6) and *Rip140−/− (n=6) mice at 12 months of age. Protein levels was normalized to γ-TUB expression and are presented as the fold change relative to levels in WT mice (right).** 

**Ex vivo mitochondrial respiration analysis performed on permeabilized spinal cord sections from 12-month-old WT (n=5), *Abcd1* (n=5), *Abcd1*/*Rip140−/− (n=6) and *Rip140−/− (n=5) mice. C.I = complex I, C.I + II = complex I + II, C.II = complex II, RCR = Respiratory control ratio.** 

**ATP content in the spinal cords of 12-month-old WT (n=8), *Abcd1* (n=6), *Abcd1*/*Rip140−/− (n=9) and *Rip140−/− (n=10) mice. The data are shown as the fold change compared to the level in WT mice.** 

**NADH and NAD+ content in the spinal cords of 12-month-old WT (n=10), *Abcd1* (n=9), *Abcd1*/*Rip140−/− (n=9) and *Rip140−/− (n=9) mice. The data are shown as the fold change compared to the level in WT mice. The values are presented as the means ± SDs. (a-e, g) *p*<0.05; **p**<0.01; ***p**<0.001; two-way ANOVA followed by Tukey’s *post hoc* test. **Nonparametric Kruskal-Wallis test followed by Dunn’s *post hoc* test.** **Figure 4 RIP140 deletion prevents NFKB pathway activation and normalizes inflammatory gene expression in the spinal cords of X-ALD mice**

**Immunoblots showing pSer536-p65, p65, p50 (NFKB1), p52 (NFKB2), pIkBa, IkBa, pIKK (A/B), IKKa, and IKKb protein levels in whole spinal cord lysates from 12-month-old WT (n=6), *Abcd1* (n=6), *Abcd1*/*Rip140−/− (n=6) and *Rip140−/− (n=6) mice. γ-TUB was used as a loading control. **Expression of pro-inflammatory (*Ccl5, Ccr6, Cxcl9, Cxcl10, Il6,*
Nfkb2, Tnfa, and Tnfrsf1a) and c) anti-inflammatory (Arg1, Chil3, Fizz1, Igf1, and Tgfb1) genes in the spinal cords of WT (n=12), Abcd1− (n=12), Abcd1−/Rip140−/− (n=12) and Rip140−/− (n=12) mice at 12 months of age. Gene expression was normalized to Rplp0 expression and is presented as the fold change compared to the level in WT mice. The values are presented as the means ± SDs. * p<0.05; ** p<0.01; *** p<0.001; two-way ANOVA followed by Tukey’s post hoc test.

Figure 5 Loss of RIP140 halts axonal degeneration in Abcd1−/Abcd2−/− mice.

Immunohistological analysis of axonal pathology in 18-month-old WT, Abcd1−/Abcd2−/−, Abcd1−/Abcd2−/−/Rip140−/− and Rip140−/− mice (n=5 per genotype). Spinal cord sections were processed for (a-d) Iba1, (e-h) GFAP, (i-l) Synaptophysin, (m-p) APP, (q-t) Sudan black, (u-x) MDA, (y-b′) SMI-32 and (c′-f′) Cyt C staining. Representative images of sections from (a, e, i, m, q, u, y, c′) WT, (b, f, j, n, r, v, z, d′) Abcd1−/Abcd2−/−, (c, g, k, o, s, w, a′, e′) Abcd1−/Abcd2−/−/Rip140−/− and (d, h, l, p, t, x, b′, f′) Rip140−/− mice are shown. Scale bars = 125 μm (a-t) and 100 μm (u-f′). g′) Quantification of synaptophysin and APP accumulation and the densities of GFAP+ and Iba1+ cells (cells/mm²) in spinal cord longitudinal sections. The values are presented as the means ± SDs. * p<0.05; ** p<0.01; *** p<0.001; two-way ANOVA followed by Tukey’s post hoc test.

Figure 6 Loss of RIP140 prevents behavioural abnormalities in Abcd1−/Abcd2−/− mice.

Behavioural tests were performed on WT (n=13), Abcd1−/Abcd2−/− (n=16-17), Abcd1−/Abcd2−/−/Rip140−/− (n=12) and Rip140−/− (n=10) mice at 12 and 18 months of age. a) The clasping (12 months), b) clasping (18 months), c) bar-cross (12 months), d) bar-cross (18 months), e) treadmill (12 months) and f) treadmill (18 months) tests. The Y-axis is the a-b) clasping score, c-d) number of slips and time (in seconds) spent crossing the bar, and e-f) number of
shocks and time of shocks (in seconds) in the treadmill test (from 30 s to 7 minutes and 30 s).

The values are presented as the means ± SEMs. * p<0.05; ** p<0.01; *** p<0.001; two-way ANOVA followed by Tukey’s post hoc test. $ p<0.05; $$ p<0.01; $$$ p<0.001; the non-parametric Kruskal-Wallis test followed by Dunn’s post hoc test.

Figure 7. Model recapitulating interplay of redox and inflammatory homeostasis controlled by RIP140 in X-ALD

This study reports that RIP140 is upregulated in the CNS of the X-ALD mouse model and most severe cALD patients. RIP140 induction is mediated by excess hexacosanoic acid, diagnostic disease hallmark, through a redox-dependent mechanism involving mitochondrial ROS production. In turn, RIP140 ignites a proinflammatory reponse including TNF$\alpha$, reported to produce mitochondrial ROS, in a “devil circle” situation which may apply to other neurodegenerative or metabolic diseases. Ablation of RIP140 in X-ALD mice protects against mitochondrial and bioenergetic failure and chronic inflammation, thus preserving axonal health.
161x109mm (600 x 600 DPI)
Supplementary methods

Generation of RIP140 knockout mice with eGFP knock-in

The conditional allele was based on the Cre-mediated inversion of the sequence between the lox66 and lox71 sites. The inversion was used to disrupt the exon 4 splice acceptor sequence and introduce a gene-trap cassette that consisted of a splice acceptor sequence, the eGFP coding sequence, and a polyadenylation signal. Therefore, a lox66 site was placed in intron 3 and a lox71 site in exon 4, upstream of the start codon in the untranslated region of the gene. The gene-trap cassette was in the reverse orientation relative to transcription of the RIP140 gene. The PGK-neo selection cassette was inserted between the gene-trap cassette and exon 4. The PGK-neo cassette was flanked by FRT sites and was deleted using FLP recombinase (conditional allele (neo removed)) (Fig. S2a).

The targeting vector was constructed using three genomic fragments, i.e., the 5’ homology arm, the exon 4 splice acceptor fragment and the 3’ homology arm, all of which were generated by PCR. Both homology arms were approximately 5-6 kb in length. For the conditional allele (neo removed), splicing from exon 3 to exon 4 occurred normally. The splice acceptor and the polyA signal in the gene-trap cassette were not recognized because they were in an inverted orientation (Fig. S2a). Cre-mediated recombination between the lox66 and lox71 sites resulted in the generation of one loxP site and one double-mutant lox site (Lox DM). In the inverted locus, splicing occurred from exon 3 to the splice acceptor in the gene-trap cassette, resulting in the expression of eGFP (Fig. S2a).

The specific primers used for genotyping genomic DNA from mouse tail tips were as follows: a common forward primer (5’-TGAGCGTTGTGGATGA-3’) used with a specific reverse primer for the WT (5’-ATAGCAGTCCCGGCGAGATC-3’), conditional (5’-CCACTACCTGAGCACCAGT-3’) or KO allele (5’-TGGATGTGGCCTGTCATGT-3’). These combinations of primers amplified a 504 bp segment for the conditional allele, a 704 bp segment for the WT allele, and a 530 bp segment for the KO allele (Fig. S2b) under the following PCR conditions: 94°C for 30 s, 54°C for 30 s, 72°C for 60 s (WT and conditional alleles) or 94°C for 30 s, 62°C for 30 s, 72°C for 90 s (knockout allele).
**Supplementary figure legends**

**Fig. S1** RIP140 expression in the X-ALD mouse model

**a**) Representative immunoblots (left) showing RIP140 protein levels in the cerebral cortex and livers of WT (n=8) and Abcd1−/− (n=8) mice at 12 months of age. Protein levels were normalized to γ-TUB expression and are presented as the fold change relative to the level in WT mice (right). The values are presented as the means ± SDs. **b**) RIP140 mRNA expression was measured by quantitative RT-PCR in organotypic spinal cord slice cultures (OSCSCs) from WT and Abcd1−/− mice treated with H2O2 (10 µM) or vehicle (WT n=6; WT + H2O2 n=5; Abcd1−/− n=7; Abcd1−/− + H2O2 n=3). Gene expression was normalized relative to Rplp0 expression and is presented as the fold change compared to the level in vehicle-treated WT OSCSCs. **c**) and **d**) RIP140 mRNA expression was measured by quantitative RT-PCR in **c**) WT and **d**) Abcd1−/− OSCSCs treated with C26:0 (100 µM) and/or Trolox (500 µM) compared with vehicle-treated OSCSCs (WT n=6 per condition; Abcd1−/− n=5 per condition). Gene expression was normalized relative to Rplp0 expression and is presented as the fold change compared to the level in vehicle-treated OSCSCs. The values are presented as the means ± SDs. * p<0.05; ** p<0.01; *** p<0.001; **) two-way or (c, d) two-way ANOVA followed by Tukey’s *post hoc* test.
**Fig. S2** Generation and characterization of RIP140 knockout mice.

(a) Schematic of the different alleles inserted into the *Rip140* gene locus. The conditional allele contains a lox66 and a lox71 site placed in intron 3 and in exon 4, respectively. A gene-trap cassette consisting of the exon 4 splice acceptor (SA), EGFP coding sequence and the BGH polyA (pA) signal was placed in intron 3 within the floxed region. The gene-trap cassette was in the reverse orientation relative to transcription of the *Rip140* gene. The PGK-neo selection cassette was inserted between the gene-trap cassette and exon 4. Moreover, the PGK-neo cassette was flanked by FRT sites. To generate the conditional allele (neo removed), conditional mice were crossed with FLPe recombinase mice to delete the PGK-neo cassette. In addition, splicing from exon 3 to exon 4 occurred normally. The splice acceptor and the polyA signal in the gene-trap cassette were not recognized because they were in the inverted orientation. Cre-mediated recombination between the lox66 and lox71 sites resulted in the generation of one loxP site and one double-mutant lox site. In the inverted locus, splicing occurred from exon 3 to the splice acceptor in the gene-trap cassette, resulting in the expression of EGFP (Cre-mediated inverted allele).

(b) PCR bands obtained after genotyping wild-type and knockout mice. The WT allele was 704 bp and the KO allele was 530 bp.

(c) Western blot analysis of RIP140 and y-TUB expression in different tissues. WT and KO samples are shown for spinal cord, hippocampus, cerebellum, BAT, WAT, and liver.
(Rip140<sup>+/+</sup>), heterozygous (Rip140<sup>+/−</sup>) and knockout (Rip140<sup>−/−</sup>) mice. c) RIP140 protein levels were measured in the spinal cords, hippocampus, cerebellum, brown adipose tissue (BAT), white adipose tissue (WAT), and livers of wild-type (WT) and Rip140<sup>−/−</sup> (KO) mice. γ-TUB was used as a loading control.

**Fig. S3** RIP140 localization in the adult mouse central nervous system (corresponding to Figure 2).

Immunofluorescence study of RIP140 localization, as determined coimmunostaining for GFP and different cell type markers, in the central nervous system of 1-month-old Rip140<sup>−/−</sup> mice. Representative images of double immunofluorescence for green-labelled GFP-positive cells (GFP antibody) and red-labelled (a-d) astrocytes (GFAP), (e-g) neurons (NeuN), (h-j) microglial cells (Iba1), and k) Olig2<sup>+</sup> cells (Olig2) are shown. The region of the CNS is indicated in each image. The white arrows indicate cells double-positive for GFP (green) and the corresponding cell type marker (red). The insets show green, red and merged signals at 2.5x (a-j) or 4.75x magnification (k). Scale bar = 100 µm.
mtDNA content was measured in the spinal cords, gastrocnemius muscles, soleus muscles, BAT, cerebral cortex, cerebellum, hippocampus, pons, white adipose tissue (WAT), livers, and hearts of 3-month-old WT (n=5) and Rip140-/- (n=4) mice. mtDNA content is expressed as the ratio of mtDNA (Cytb) to nDNA (Cebpa), and is presented as the fold change compared to the level in WT mice. The values are presented as the means ± SDs. * p<0.05; unpaired Student’s t-test.

The protein level was normalized to γ-TUB expression and is presented as the fold change relative to the level in WT. The values are presented as the means ± SDs. * p<0.05; ** p<0.01; *** p<0.001; two-way ANOVA followed by Tukey’s post hoc test.
Supplementary Table 1

List of antibodies used in this study

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## Supplementary Table 2

List of human brain samples used in this study

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**Supplementary Table 3**

List of Q-PCR probes used in this study

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## Supplementary Table 4

Scoring for the clasping test

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### Supplementary Table 5

List of exact p values reported in this article

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