

# Oxidative stress modulates mitochondrial failure and cyclophilin D function in X-linked adrenoleukodystrophy

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A common process associated with oxidative stress and severe mitochondrial impairment is the opening of the mitochondrial permeability transition pore, as described in many neurodegenerative diseases. Thus, inhibition of mitochondrial permeability transition pore opening represents a potential target for inhibiting mitochondrial-driven cell death. Among the mitochondrial permeability transition pore components, cyclophilin D is the most studied and has been found increased under pathological conditions. Here, we have used *in vitro* and *in vivo* models of X-linked adrenoleukodystrophy to investigate the relationship between the mitochondrial permeability transition pore opening and redox homeostasis. X-linked adrenoleukodystrophy is a neurodegenerative condition caused by loss of function of the peroxisomal ABCD1 transporter, in which oxidative stress plays a pivotal role. In this study, we provide evidence of impaired mitochondrial metabolism in a peroxisomal disease, as fibroblasts in patients with X-linked adrenoleukodystrophy cannot survive when forced to rely on mitochondrial energy production, i.e. on incubation in galactose. Oxidative stress induced under galactose conditions leads to mitochondrial damage in the form of mitochondrial inner membrane potential dissipation, ATP drop and necrotic cell death, together with increased levels of oxidative modifications in cyclophilin D protein. Moreover, we show increased expression levels of cyclophilin D in the affected zones of brains in patients with adrenomyeloneuropathy, in spinal cord of a mouse model of X-linked adrenoleukodystrophy (*Abcd1*-null mice) and in fibroblasts from patients with X-linked adrenoleukodystrophy. Notably, treatment with antioxidants rescues mitochondrial damage markers in fibroblasts from patients with X-linked adrenoleukodystrophy, including cyclophilin D oxidative modifications, and reverses cyclophilin D induction *in vitro* and *in vivo*. These findings provide mechanistic insight into the beneficial effects of antioxidants in neurodegenerative and non-neurodegenerative cyclophilin D-dependent disorders.

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**Keywords:** Cyclophilin D; mitochondrial permeability transition pore; neurodegenerative disease; oxidative stress; X-linked adrenoleukodystrophy

**Abbreviations:** H<sub>2</sub>-DCFDA = 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate diacetoxyethyl-ester; TMRE = tetramethylrhodamine ethyl ester

## Introduction

Oxidative stress has been shown to participate in the onset and/or progression of several human neurodegenerative diseases (Lin and Beal, 2006; Pratico, 2008; Stack *et al.*, 2008; Zhou *et al.*, 2008; Martinez *et al.*, 2010). Compelling evidence indicates that excessive levels of radical oxygen species can modify or deactivate protein activity or even alter its conformation (Wang *et al.*, 2012). Oxidative stress also provokes mutations in mitochondrial and nuclear DNA, causing mitochondrial dysfunction and cell death (Pamplona and Barja, 2007).

Mitochondria are organelles playing a pivotal role in orchestrating cell survival and death, and their optimal functioning is required to preserve cell and organism viability (Lin and Beal, 2006; Du and Yan, 2010; Fernandez-Checa *et al.*, 2010; Blackstone and Chang, 2011). Nonetheless, mitochondria are main targets of oxidative stress. In pathological conditions, there are increasing probabilities of mitochondrial permeability transition pore formation, triggering an irrevocable necrotic cell death (Petronilli *et al.*, 1994; Baines *et al.*, 2005; Halestrap, 2005; Nakagawa *et al.*, 2005). It is well established that once the mitochondrial permeability transition pore is formed, a non-selective, high conductance pore allowing the flux of solutes up to 1.5 kDa across the inner mitochondrial membrane is opened. This results in the dissipation of mitochondrial membrane potential and damage to the mitochondrial respiratory chain, thereby reducing ATP production and exacerbating mitochondrial dysfunction. Moreover, mitochondrial osmotic swelling leads to ruptures in outer mitochondrial membrane triggering irreversible cell death (Baines *et al.*, 2005; Bernardi *et al.*, 2006; Du and Yan, 2010). Although the protein composition of mitochondrial permeability transition pore remains elusive, different components have been proposed, including the voltage-dependent anion channel, the adenine nucleotide translocator, the mitochondrial phosphate carrier and the cyclosporin A target cyclophilin D (Halestrap, 2009). Genetic ablation studies have confirmed that cyclophilin D is an essential component of mitochondrial permeability transition pore (Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005), although formal proof is lacking for the rest of the above-mentioned candidates. In this regard, blockade of cyclophilin D by genetic abrogation or pharmacological inhibition protects mitochondria in many age-related neurodegenerative disease models, such as Alzheimer's disease (Du *et al.*, 2008), Parkinson's disease (Gandhi *et al.*, 2009; Thomas *et al.*, 2011), amyotrophic lateral sclerosis (Karlsson *et al.*, 2004; Martin *et al.*, 2009; Martin, 2010) and multiple sclerosis (Forte *et al.*, 2007).

Oxidative stress is a well-established inducer of mitochondrial permeability transition pore formation (Baines *et al.*, 2005; Halestrap, 2005; Du *et al.*, 2008; Martin *et al.*, 2009), but little is known about the molecular mechanisms involved in this process.

For this reason, we chose to study cellular and *in vivo* models of X-linked adrenoleukodystrophy (McKusick No.300100), a rare neurometabolic disease in which oxidative stress is the main early contributing pathogenic factor (Fourcade *et al.*, 2008). X-linked adrenoleukodystrophy is the most frequent peroxisomal disorder (minimum incidence 1:17 000 males) characterized by progressive demyelination within the CNS, adrenal insufficiency and a pathognomonic accumulation of very long-chain fatty acids (C ≥ 22:0) in plasma and tissues, in particular hexacosanoic acid, C26:0 (Powers *et al.*, 2000; Moser, 2001; Ferrer *et al.*, 2010). This inheritable disorder is caused by mutations in the *ABCD1* gene encoding the homonymous ATP-binding cassette peroxisomal transporter, which is involved in the import of very long-chain fatty acids, and very long-chain fatty acids-coenzyme A esters into the peroxisome for degradation (Hettema *et al.*, 1996; van Roermund *et al.*, 2008).

Classical inactivation of *Abcd1* in the mouse results in late onset neurodegeneration with axonopathy in spinal cord, in the absence of inflammatory demyelination in the brain, resembling the most frequent X-linked adrenoleukodystrophy phenotype or adrenomyeloneuropathy (Forss-Petter *et al.*, 1997; Lu *et al.*, 1997; Pujol *et al.*, 2002, 2004). Oxidative damage has been shown in post-mortem brain samples from individuals with cerebral X-linked adrenoleukodystrophy (Gilg *et al.*, 2000) and in mouse spinal cords well before disease onset (Fourcade *et al.*, 2008). Further, we recently reported that a combination of antioxidants halts clinical progression and reverses axonal damage in the X-linked adrenoleukodystrophy mouse model, thereby providing formal conceptual proof that oxidative injury is a major aetiopathogenic factor in this disease (Singh and Pujol, 2010; Lopez-Erauskin *et al.*, 2011; Galea *et al.*, 2012). These findings are currently being translated in a clinical trial for patients with adrenomyeloneuropathy (NCT01495260).

Our present study uncovers a functional impairment of mitochondria in X-linked adrenoleukodystrophy fibroblasts, underscoring the essential role of mitochondrial permeability transition pore in cell death. Further, we present novel molecular events underlying oxidative stress-induced mitochondrial permeability transition pore formation in X-linked adrenoleukodystrophy and molecular actions of antioxidants on cyclophilin D. This highlights the rationale for using these types of agents as a therapeutic option against mitochondrial permeability transition pore opening, a cardinal event observed in most prevalent neurodegenerative diseases.

## Materials and methods

### Chemicals

The following chemicals were used: 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate diacetoxyethyl-ester (H<sub>2</sub>-DCFDA; Invitrogen),

L-Buthionine-sulfoximine (Sigma), cyclosporin A (Calbiochem), FK506 (LC Laboratories), Hoechst 33342 (Sigma), monochlorobimane (Molecular Probes), *N*-acetylcysteine (Sigma), staurosporine (Sigma), tetramethylrhodamine ethyl ester (TMRE; Invitrogen) and Z-VAD, (Sigma).

## Antibodies

The following antibodies were used for western blots: anti-caspase 3, dilution 1:1000, incubated overnight at 4°C (Cell Signaling catalogue number 9661 and 9662); cyclophilin D antibody (MitoScience) 1:1000 dilution; anti-gamma-tubulin [T6557, clone GTU-88 (Sigma)], dilution: 1:5000, incubated 1 h at room temperature; goat anti-rabbit Immunoglobulin G linked to horseradish peroxidase, dilution: 1:10 000 [81–6520 (Invitrogen)]; and goat anti-mouse Immunoglobulin G linked to horseradish peroxidase, dilution: 1:10 000 [81–6120 (Invitrogen)] used as secondary antibodies and incubated 1 h at room temperature.

## Cell cultures and treatments

Human control and X-linked adrenoleukodystrophy fibroblasts were obtained after informed consent at the Bellvitge University Hospital. Cells were grown in Dulbecco's modified Eagle medium containing 10% foetal bovine serum, 100 U/ml penicillin and 100 mg streptomycin, at 37°C in humidified 95% air/5% CO<sub>2</sub>, to 80–90% confluence. To perform our experiments, Dulbecco's modified Eagle medium without D-glucose, pyruvate or L-glutamine was used. Cells were cultured in this medium supplemented with 1 g/l of glucose or 1 g/l of galactose and 10% foetal bovine serum. In these conditions, different drug effects were tested: general caspase inhibitor Z-VAD (1 μM), cyclosporin A (5 μM), FK506 (5 μM), *N*-acetylcysteine (1 mM), L-Buthionine-sulfoximine (500 μM) and staurosporine (1 μM for 14 h).

## Evaluation of intracellular radical oxygen species

Intracellular radical oxygen species levels were estimated using the radical oxygen species-sensitive H<sub>2</sub>DCFDA probe as described (Fourcade *et al.*, 2008). Following incubation with 10 μM H<sub>2</sub>DCFDA for 30 min, cells were washed twice with PBS and lysated with 1% Triton<sup>TM</sup>. The fluorescence of H<sub>2</sub>DCFDA-stained cells was measured with a spectrofluorimeter (excitation wavelength 493 nm, emission wavelength 527 nm).

## Adenosine triphosphate measurement

At different time points (12, 24, 36 and 40 h) after changing the medium to galactose, ATP levels were measured by a chemiluminescence system using ATPlite 1step (PerkinElmer), according to the manufacturer's protocol. ATP levels were normalized by total protein concentration.

## Cell death measurements

After the indicated treatments, human fibroblasts viability was quantified using CytoTox-ONE<sup>TM</sup> Homogeneous Membrane Integrity Assay (Promega), according to the manufacturer's instructions.

## Nuclear chromatin condensation

X-linked adrenoleukodystrophy fibroblasts nuclei were stained with Hoechst 33342 (10 μg/ml) as described (Lizard *et al.*, 1995), and the nuclei were observed using confocal microscopy.

## Evaluation of reduced glutathione

Reduced glutathione was estimated using monochlorobimane (excitation wave length 360 nm, emission wave-length 460 nm) as described (Fourcade *et al.*, 2008).

## Inner mitochondrial membrane potential quantification by flow cytometry

Treated cells were washed with PBS and incubated with 50 nM of TMRE (Molecular Probes) in pre-warmed PBS for 30 min at 37°C. Cells were trypsinized, centrifuged at 1000 g for 5 min and resuspended in pre-warmed PBS. All samples were captured in a FACSCanto<sup>TM</sup> recording 20 000 cells for each condition and genotype tested. Histograms showing the percentage of depolarized cells were obtained after gating live cells. Data were analysed with FlowJo Tree Star software.

## Mono-dimensional electrophoresis and western blotting

After the required treatments, control and X-linked adrenoleukodystrophy fibroblast samples were trypsinized and washed twice with PBS. Tissues samples were removed from euthanized mice and flash frozen in liquid nitrogen. Frozen tissue samples were homogenized in radio-immunoprecipitation assay buffer using a motor-driven grinder (Sigma-Aldrich) and then sonicated for 2 min at 4°C in an ultrasonic processor UP50H (Hielscher-Ultrasound Technology). Fibroblast samples were also sonicated for 2 min at 4°C in the ultrasonic processor UP50H. Both kinds of samples were centrifuged at 1000g for 10 min at 4°C, and supernatant protein concentration was measured with BCA Protein Assay Reagent (Pierce/Thermo). Samples (10–100 μg) were loaded onto each lane of 10–15% polyacrylamide gels for 70 min at 120 mV. Resolved proteins were transferred to nitrocellulose. Secondary antibody for protein detection was conjugated with horseradish peroxidase, detected with chemiluminescence enhancer Luminol and followed by exposure to CL-XPosure<sup>TM</sup> Film (Thermo Scientific). Autoradiographs were scanned and quantified using GS800 densitometer (Bio-Rad).

## Electron microscopy

The medium and the cells cultured in the required conditions were collected and centrifuged at 1500g for 10 min to obtain the pellet of all the cells in the culture. The pellets were washed with PBS and centrifuged again at 1500g for 10 min. Supernatants were discarded and pellets were fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M PBS for 1 h at room temperature. The pellets were washed three times with 0.1 M PBS and maintained in paraformaldehyde 2% in 0.1 M PBS and then post-fixed by immersion in 1% osmium tetroxide, embedded in EPON-812 and cut with an ultramicrotome. Ultrathin sections were stained with toluidine blue, and finally selected sections were collected on copper grids and stained with uranyl acetate and Reynold's lead citrate. Sections were directly visualized with a Jem-1011 transmission electron microscope (Jeol).

## Bidimensional electrophoresis and western blotting

Redox proteomics for the identification of oxidized cyclophilin D in human X-linked adrenoleukodystrophy fibroblasts were performed as previously described (Galino *et al.*, 2011) with some modifications. After the first dimension steps, the equilibrated strips were loaded onto 15% SDS-PAGE gels (20 × 20 cm) and run at 250 V for 4.5 h at room temperature. Bidimensional gels were performed in parallel, one for Coomassie staining and the other to be transferred to nitrocellulose membrane. To detect oxidized proteins (oxyblot), the membrane was incubated with antibody against 2,4-dinitrophenylhydrazine (D9659, Sigma, dilution 1:500) (Robinson *et al.*, 1999). For immunodetection of cyclophilin D, the same membrane was incubated with antibody against cyclophilin D (Molecular Probes). For Coomassie staining, bidimensional gels were fixed for 1 h in a 30% ethanol/70% glacial acetic acid solution. Then, the fixing solution was replaced with Coomassie solution (Brilliant Blue R, B0149, Sigma) and incubated overnight at room temperature. After gel washing, the cut spots were digested, and peptides were analysed with mass spectrometry as previously reported (Galino *et al.*, 2011). The Swiss-Prot database was used to identify proteins from a spectrum generated by liquid chromatography coupled to tandem mass spectrometry (Supplementary Table S1). Briefly, we applied a taxonomy filter (*Homo sapiens*) and obtained 20 407 sequences that were used to match with our experimental data using a significance threshold  $P < 0.05$ . We obtained an identity score of 34, which lies under the score provided by the liquid chromatography coupled to tandem mass spectrometry (46.5), thus providing high reliability to the identification.

## Human brain samples

Brain tissues from patients with cerebral adrenomyeloneuropathy and healthy age matched male control subjects were obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, as described (Schluter *et al.*, 2012). Informed written consent was obtained from all patients or their legal representatives, and the local ethics committee approved the studies.

## Mouse breeding

The generation and genotyping of *Abcd1*<sup>-</sup> mice has previously been described (Lu *et al.*, 1997; Pujol *et al.*, 2002, 2004). Mice used for experiments were of a pure C57BL/6J background and were all male. Animals were sacrificed, and tissues were recovered and conserved at -80°C. All methods used in this study were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85–23, revised 1996) and with the ethical committee of The Bellvitge Institute of Biomedical Research and the government of Catalonia.

## Treatment of mice

$\alpha$ -Lipoic acid (0.5% w/w) was mixed into AIN-76A chow from Dyets. *N*-acetylcysteine (1%) was dissolved in water (pH 3.5) (Lopez-Erauskin *et al.*, 2011). Eight-month-old animals were randomly assigned to one of the following dietary groups for 4 months. Group I: wild-type mice ( $n = 8$ ) received only normal AIN-76A chow; Group II: *Abcd1*<sup>-</sup> mice ( $n = 8$ ) received only normal AIN-76A chow; and Group III: *Abcd1*<sup>-</sup> + antioxidant mice ( $n = 6$ ) were treated with chow containing  $\alpha$ -lipoic acid and with *N*-acetylcysteine in drinking water (Lopez-Erauskin *et al.*, 2011).

## Statistical analysis

Data are given as mean  $\pm$  standard deviation. Significant differences were determined by one-way ANOVA followed by Tukey's Honestly Significant Difference post-test after verifying normality ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) or Student's *t*-test ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ). Statistical analyses were performed using SPSS 12.0 program (SPSS Inc.).

## Results

### X-linked adrenoleukodystrophy fibroblasts display mitochondrial impairment

Mitochondrial dysfunction has been reported in a wide array of diseases characterized by oxidative stress, energy failure, inner mitochondrial membrane depolarization and cell death (Lin and Beal, 2006; Du *et al.*, 2008; Thomas *et al.*, 2011). Recent evidence obtained in our laboratory suggested energetic failure with reduced NADH and ATP levels, as a result of oxidation of key components of tricarboxylic acid and glycolysis in *Abcd1*<sup>-</sup> spinal cords (Galino *et al.*, 2011).

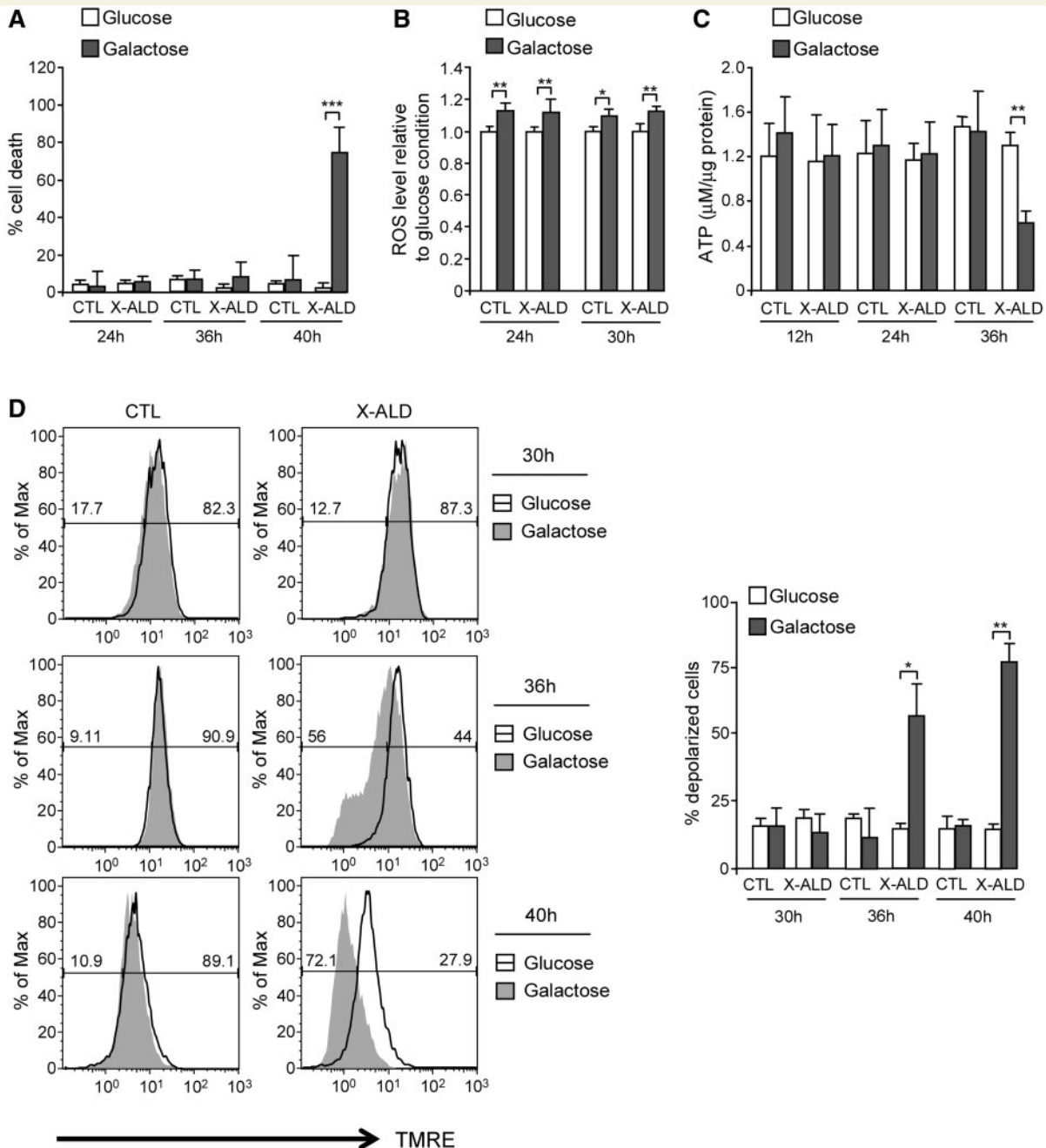
As a means to directly assess mitochondria function within intact cells, we cultured human X-linked adrenoleukodystrophy fibroblasts, usually grown in 1 g/l of glucose medium, in 1 g/l galactose medium. This is a common practice applied in molecular genetics laboratories as a diagnostic tool when a mitochondriopathy is suspected. Cells grown in glucose produce their ATP by glycolysis, largely bypassing the mitochondria. However, when the same cells are grown in media supplemented with galactose as opposed to glucose, they are forced to produce ATP through oxidative phosphorylation, as galactose is used in the glycolytic pathway at a much slower rate than glucose (Robinson *et al.*, 1992; Hofhaus *et al.*, 1996; Ghelli *et al.*, 2003; Acin-Perez *et al.*, 2009). If mitochondrial damage exists, cell viability is compromised.

Indeed, we observed that galactose induced X-linked adrenoleukodystrophy fibroblast cell death at 40 h, but not at earlier times (e.g. 24 and 36 h), indicating mitochondrial dysfunction in these cells (Fig. 1A).

To dissect the events occurring before cell death, we measured the level of radical oxygen species and ATP content as described (Fourcade *et al.*, 2008; Galino *et al.*, 2011; Lopez-Erauskin *et al.*, 2011). Also, we measured inner mitochondrial membrane potential with TMRE and analysed the results by flow cytometry. TMRE is a voltage-sensitive red–orange fluorescent indicator for mitochondrial transmembrane potential (Zhou *et al.*, 2010). Mitochondrial depolarization (disrupting or decreasing membrane potential) results in a loss of dye from the mitochondria and a decrease in fluorescence intensity.

We observed that culture in galactose provoked (i) an increase in intracellular radical oxygen species level compared with glucose conditions, both in control and X-linked adrenoleukodystrophy fibroblasts (Fig. 1B); (ii) energetic failure in X-linked adrenoleukodystrophy fibroblasts as shown by lowered levels of ATP



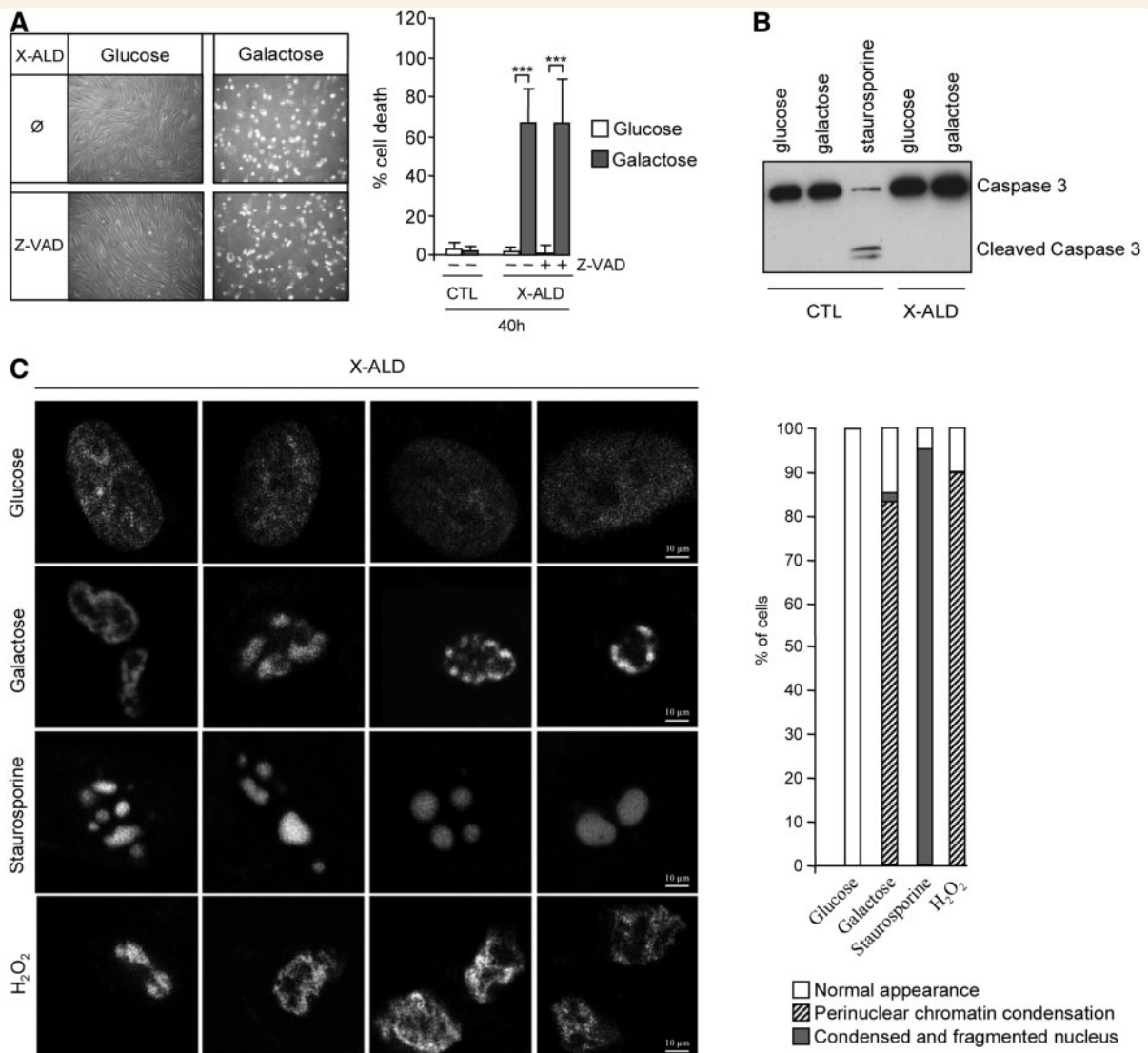


**Figure 1** Mitochondrial dysfunction in X-linked adrenoleukodystrophy fibroblasts. Cell death (A), intracellular radical oxygen species levels (B), ATP content (C) and inner mitochondrial membrane potential (D) were quantified in control and X-linked adrenoleukodystrophy fibroblasts cultured in presence of 1 g/l glucose or glucose-free medium containing 1 g/l galactose (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ;  $n = 4$ /genotype and condition). In D, the numeric values above the line correspond to the percentage of polarized cells on the right-hand and depolarized cells on the left-hand of each panel. CTL = control; ROS = radical oxygen species; X-ALD = X-linked adrenoleukodystrophy.

(Fig. 1C); and (iii) depolarization in X-linked adrenoleukodystrophy fibroblasts (Fig. 1D). All these events preceded cell death. However, once cell death was observed (40h), reactive oxygen species and ATP levels dramatically dropped (Supplementary Fig. 1A and B), and the inner mitochondrial membrane potential was completely disrupted (Fig. 1D), coinciding with a shift of the whole cell population to a smaller size region (Supplementary Fig. 1C).

## Galactose-induced cell death occurs independently of caspase activity

We next evaluated the type of cell death induced by galactose in X-linked adrenoleukodystrophy fibroblasts. To discriminate between types of cell demise, we inhibited caspase activation using the general caspase inhibitor Z-VAD. Interestingly, we observed no prevention of galactose-induced cell death (Fig. 2A). Moreover,



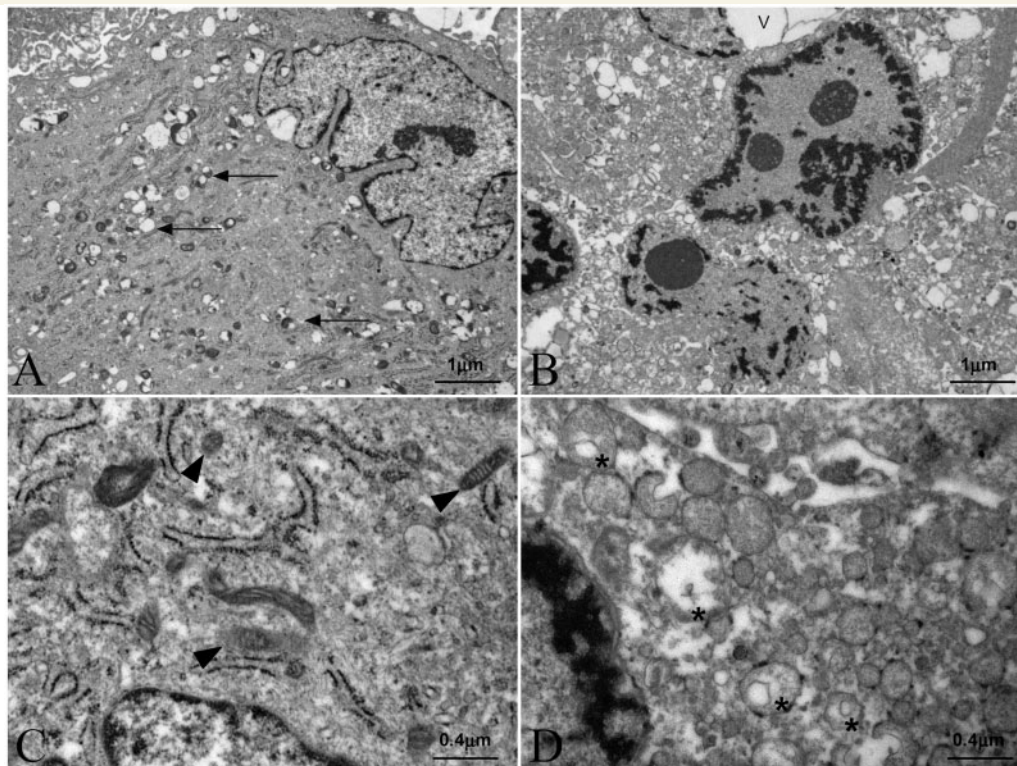
**Figure 2** Galactose induces necrotic cell death in X-linked adrenoleukodystrophy fibroblasts. Control and X-linked adrenoleukodystrophy fibroblasts cultured in glucose or galactose were treated with general caspase inhibitor Z-VAD, and cell death was measured (A). Cleaved caspase 3 was analysed in different conditions by western blot (B). Staurosporine (1 microM during 14 h) was used as a positive control of caspase 3 activator ( $***P < 0.001$ ;  $n = 4$ /genotype and condition). The pattern of chromatin condensation induced by galactose was analysed using Hoescht 33342 staining (C). H<sub>2</sub>O<sub>2</sub> (10 mM for 3 h) and staurosporine (2  $\mu$ M for 6 h) were used as positive control for necrotic and apoptotic cell death, respectively. X-linked adrenoleukodystrophy fibroblasts of six different patients were analysed, and 25 cells per condition were scored. Representative pictures and quantification are shown. CTL = control; X-ALD = X-linked adrenoleukodystrophy.

galactose treatment did not generate cleavage of caspase 3, the caspase active form, in contrast with cleavage observed with staurosporine, a classical apoptosis inducer and caspase 3 activator (Fig. 2B). Further, we examined nuclear chromatin condensation using the Hoechst 33342 dye as described (Lizard *et al.*, 1995). We thus compared galactose-induced chromatin condensation pattern with (i) H<sub>2</sub>O<sub>2</sub>-treated cells as positive control for necrotic cell death (Lee and Shacter, 2000) and (ii) staurosporine-treated cells as positive control for apoptotic cell death (Seo and Seo, 2009) (Fig. 2C). Galactose-induced nuclear morphology presented more similarities with H<sub>2</sub>O<sub>2</sub>-treated cells than with the classical apoptotic chromatin condensation phenomenon observed in staurosporine-treated cells. In addition, electron microscopy

analysis of X-linked adrenoleukodystrophy fibroblasts treated with galactose showed that these cells had features of necrosis, such as vacuolization, and non-apoptotic chromatin condensation, instead of the classical apoptotic morphology (Fig. 3A–D). Collectively, these data support the notion that galactose induces necrotic cell death in X-linked adrenoleukodystrophy fibroblasts.

## Galactose-induced cell death is oxidative stress dependent

We have previously shown that a cocktail of antioxidants, including *N*-acetylcysteine,  $\alpha$ -lipoic acid and vitamin E, or



**Figure 3** X-linked adrenoleukodystrophy fibroblasts show necrotic features during galactose-induced cell death. X-linked adrenoleukodystrophy fibroblasts were cultured in glucose (A) (shown at larger magnification in C); or in glucose-free medium containing galactose (B) (shown at larger magnification in D). After 40 h, cells were collected and analysed by electron microscopy. V = vacuole; arrows indicate lipid inclusions; arrowheads indicate morphologically normal appearing mitochondria; asterisks indicate swollen mitochondria ( $n = 4/\text{condition}$ ).

*N*-acetylcysteine and  $\alpha$ -lipoic acid alone, reversed oxidative damage to proteins and DNA, mitochondrial energetic metabolism impairment, immunohistological signs of axonal degeneration and associated locomotor disability in an X-linked adrenoleukodystrophy mouse model (Galino *et al.*, 2011; Lopez-Erauskin *et al.*, 2011).

To evaluate whether oxidative stress plays a role in galactose-induced cell death, we cultured X-linked adrenoleukodystrophy fibroblasts in the presence of galactose and the antioxidant *N*-acetylcysteine. The treatment induced cell survival (Fig. 4A), led to the recovery of normal levels of ATP (Fig. 4B), and reversed mitochondrial depolarization (Fig. 4C), demonstrating that oxidative stress plays a central role in the mitochondrial dysfunction observed under these conditions.

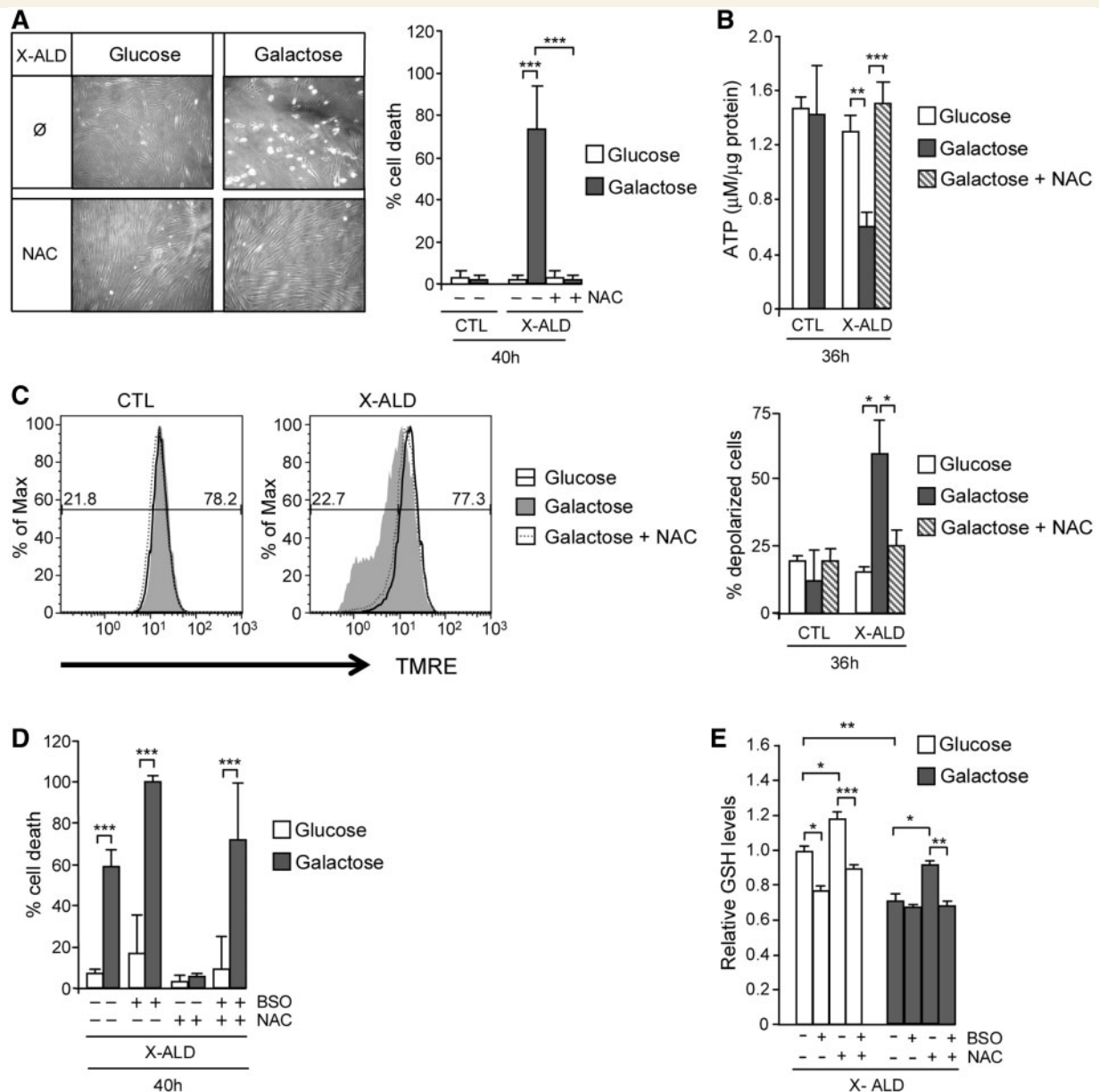
We and others have previously reported that C26:0 excess provokes decrease of the reduced glutathione (Fourcade *et al.*, 2008; Baarine *et al.*, 2012b), the major antioxidant barrier of the cell (Harvey *et al.*, 2008). Considering that *N*-acetylcysteine is the precursor of reduced glutathione, we wondered whether the positive effect observed with *N*-acetylcysteine was due to regeneration of reduced glutathione, or alternatively, due to other effects (antioxidant or other) of *N*-acetylcysteine. To answer this question, we used L-buthionine-sulfoximine, a specific inhibitor of the key enzyme of reduced glutathione synthesis, the  $\gamma$ -glutamylcysteine-synthetase enzyme, as described (Fourcade *et al.*, 2008).

Galactose-induced cell death of X-linked adrenoleukodystrophy fibroblasts was not prevented when L-buthionine-sulfoximine was applied together with *N*-acetylcysteine (Fig. 4D). Further, we measured reduced glutathione using monochlorobimane and confirmed that cell survival was correlated with reduced glutathione amount (Fig. 4E). Taken together, our data indicate that reduced glutathione synthesis is required to prevent cell death, and, thus, oxidative stress plays a paramount role in this process.

### Mitochondrial permeability transition pore opening underlies galactose-induced cell death in X-linked adrenoleukodystrophy fibroblasts

Mitochondria play a critical role in initiating both apoptotic and necrotic cell death. A major player in necrosis is the mitochondrial permeability transition pore, a non-specific pore that opens in the inner mitochondrial membrane promoting massive swelling of mitochondria, drop of membrane potential, rupture of the outer membrane and cell death (Halestrap *et al.*, 2002; Baines *et al.*, 2005; Bernardi *et al.*, 2006; Du and Yan, 2010). When mitochondria become depolarized, inhibition of oxidative phosphorylation and stimulation of ATP hydrolysis occur (Du *et al.*, 2008; Halestrap, 2009).





**Figure 4** X-linked adrenoleukodystrophy fibroblast cell death and metabolic failure in galactose is oxidative stress dependent. Control and X-linked adrenoleukodystrophy fibroblasts cultured in glucose or galactose were treated with 1 mM of *N*-acetylcysteine. Cell death (A), ATP content (B) and inner mitochondrial membrane potential (C) were measured. In C, the numeric values above the line correspond to the percentage of polarized cells on the right-hand and depolarized cells on the left-hand of each panel. Fibroblasts cell death was quantified under the same conditions with L-buthionine-sulfoximine (500  $\mu$ M) treatment (D). Reduced glutathione amount was quantified fluorimetrically using monochlorobimane in X-linked adrenoleukodystrophy fibroblasts cultured in glucose or in galactose under *N*-acetylcysteine and/or L-buthionine-sulfoximine treatments (E) (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ;  $n = 4$ /condition). BSO = L-buthionine-sulfoximine; CTL = control; GSH = reduced glutathione; NAC = *N*-acetylcysteine; X-ALD = X-linked adrenoleukodystrophy.

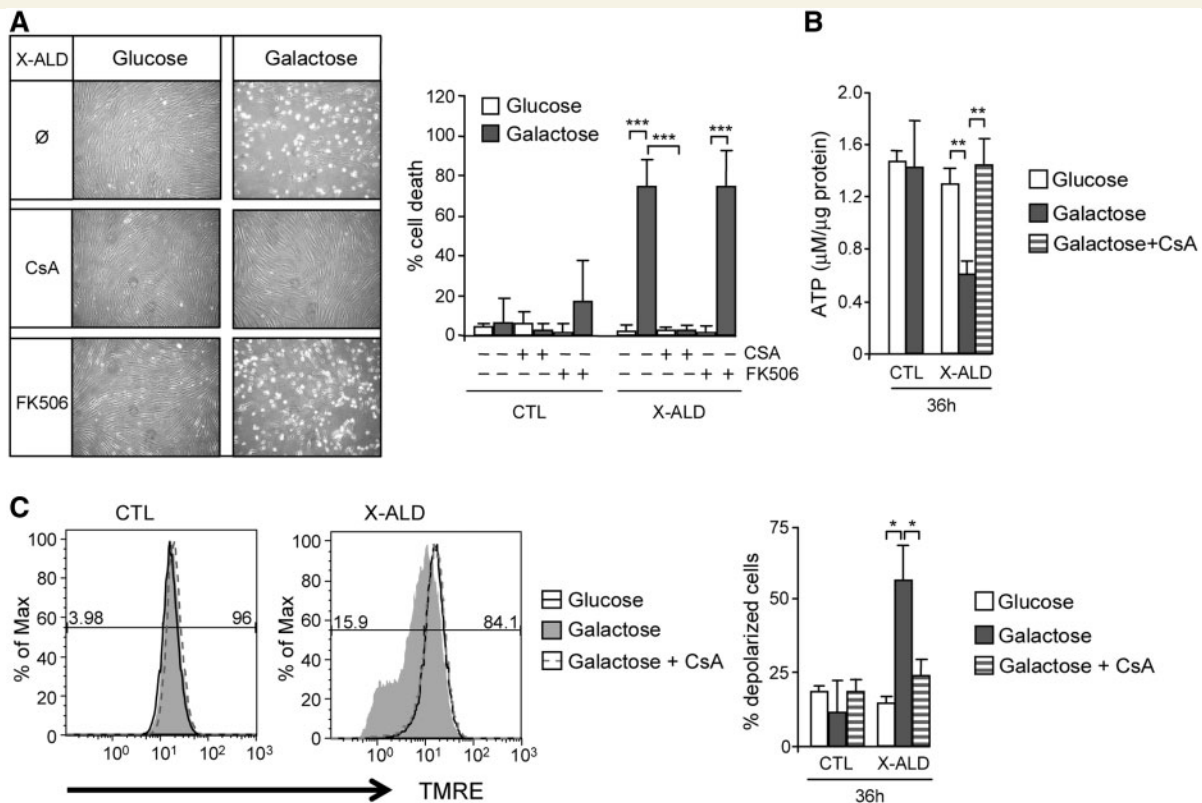
To study a potential implication of mitochondrial permeability transition pore opening in galactose-induced cell death in X-linked adrenoleukodystrophy fibroblasts, we used cyclosporin A, a clinically used immunosuppressive agent, which among other properties, acts to inhibit mitochondrial permeability transition pore opening through its binding to mitochondrial matrix protein cyclophilin D (Crompton *et al.*, 1988; Halestrap and Davidson, 1990).

Cyclophilin D is the only existent mitochondrial peptidyl-prolyl cis-trans isomerase that under pathological conditions is

translocated to the inner mitochondrial membrane, where it is perceived to interact with the adenine nucleotide translocator inducing pore formation (Connern and Halestrap, 1994; Crompton *et al.*, 2002; Halestrap *et al.*, 2002; Zamzami *et al.*, 2005; Halestrap, 2006).

We observed that cyclosporin A prevented galactose-induced X-linked adrenoleukodystrophy fibroblasts cell death (Fig. 5A). However, considering that cyclosporin A can also inhibit calcineurin, we used the compound FK506 that inhibits calcineurin





**Figure 5** Galactose-induced mitochondrial dysfunction and cell death in X-linked adrenoleukodystrophy fibroblasts is caused by mitochondrial permeability transition pore opening. Control and X-linked adrenoleukodystrophy fibroblasts cultured in glucose or galactose were treated with 5 μM of cyclosporin A or 5 μM of FK506, and cell death was measured (A). In the same conditions with cyclosporin A treatment, ATP content (B) and inner mitochondrial membrane potential (C) were quantified (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ;  $n = 4$ /genotype and condition). In C, the numeric values above the line correspond to the percentage of polarized cells on the right-hand and depolarized cells on the left-hand of each panel. CsA = cyclosporin A; CTL = control; X-ALD = X-linked adrenoleukodystrophy.

activity, but shows no binding to cyclophilin D, as a negative control (Friebert *et al.*, 1998). The treatment of X-linked adrenoleukodystrophy fibroblasts with FK506 compound did not prevent galactose-induced cell death, indicating that induction of cell death occurs through mitochondrial permeability transition pore formation (Fig. 5A).

In addition, the presence of cyclosporin A counteracts reduction in ATP (Fig. 5B) and inner mitochondrial membrane potential dissipation (Fig. 5C) induced by galactose in X-linked adrenoleukodystrophy fibroblasts. Moreover, we observed mitochondrial swelling in galactose-treated X-linked adrenoleukodystrophy fibroblasts by electron microscopy preparations (Fig. 3B and D). Taken together, these data indicate that mitochondrial permeability transition pore opening underlies the cell death of X-linked adrenoleukodystrophy fibroblasts on galactose.

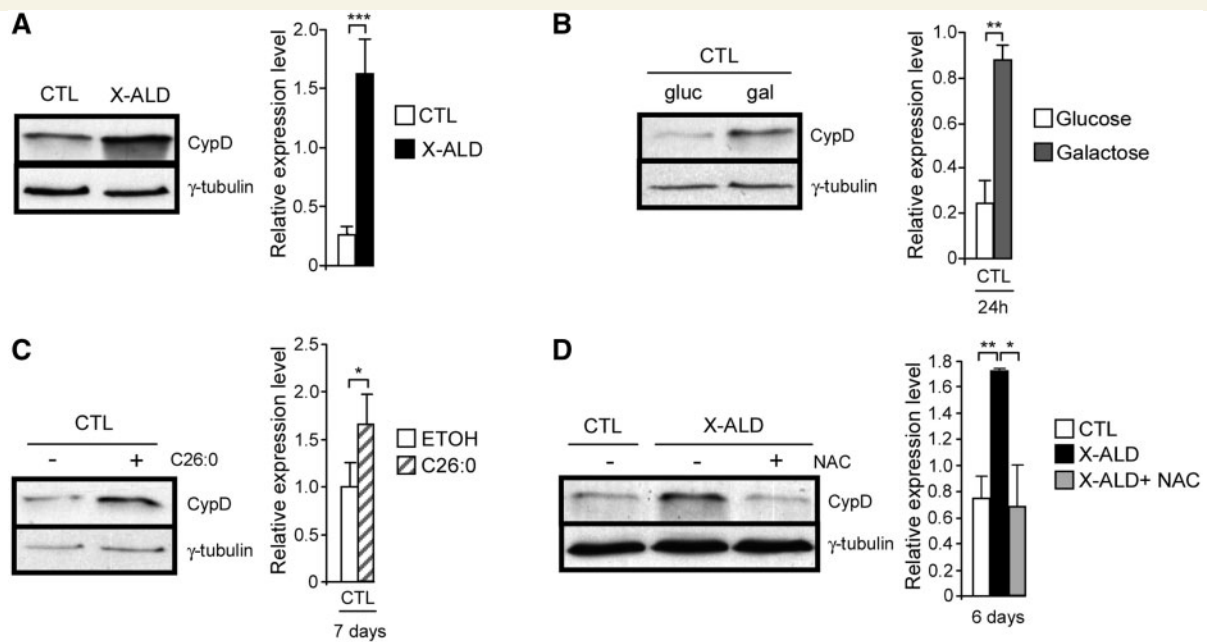
## Cyclophilin D expression is modulated by oxidative stress in X-linked adrenoleukodystrophy *in vitro* and *in vivo*

Cyclophilin D is the only firmly established mitochondrial permeability transition pore component and plays an essential role in its

formation (Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). As accumulation of cyclophilin D under pathological conditions in several disorders and in ageing has been described (Du *et al.*, 2008; Gandhi *et al.*, 2009; Martin, 2010), we decided to study cyclophilin D expression in X-linked adrenoleukodystrophy fibroblasts. We found more marked cyclophilin D expression in X-linked adrenoleukodystrophy compared with control fibroblasts at baseline levels, when cells were cultured in glucose (Fig. 6A). Galactose incubation (Fig. 6B) and incubation with the very long-chain fatty acid C26:0 (Fig. 6C) induced cyclophilin D overexpression in control fibroblasts. Treatment with the antioxidant *N*-acetylcysteine reduced cyclophilin D to control levels in X-linked adrenoleukodystrophy fibroblasts (Fig. 6D).

## Cyclophilin D is oxidized in X-linked adrenoleukodystrophy human fibroblasts, and *N*-acetylcysteine counteracts this effect

We earlier reported an increase in oxidized proteins in *Abcd1*<sup>-/-</sup> mice spinal cord and in X-linked adrenoleukodystrophy fibroblasts (Fourcade *et al.*, 2008; Galino *et al.*, 2011; Lopez-Erauskin *et al.*, 2011). Wondering whether cyclophilin D could also be oxidized in



**Figure 6** Cyclophilin D expression is modulated by oxidative stress. Cyclophilin D levels were quantified in control and X-linked adrenoleukodystrophy fibroblasts at basal levels (A), in control fibroblasts after culture in galactose for 24 h (B) and with C26:0 (50  $\mu$ M) for 7 days (C), and after treating X-linked adrenoleukodystrophy fibroblasts with *N*-acetylcysteine (0.5 mM) for 6 days (D) (\* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001;  $n$  = 5/genotype and condition). CTL = control; CypD = cyclophilin D; ETOH = ethanol; NAC = *N*-acetylcysteine; X-ALD = X-linked adrenoleukodystrophy.

X-linked adrenoleukodystrophy, we performed redox proteomic experiments with human fibroblasts. We observed a significant increase in cyclophilin D oxidation in X-linked adrenoleukodystrophy fibroblasts (Fig. 7A and B). Interestingly, antioxidant treatment with *N*-acetylcysteine reversed cyclophilin D oxidation (Fig. 7A and B). These data suggest a plausible molecular mechanism underlying the beneficial action of antioxidants in mitochondrial permeability transition pore opening, which deserves further testing in relevant models (Petrosillo *et al.*, 2009; Greco *et al.*, 2011; Loor *et al.*, 2011).

### Cyclophilin D expression is increased in patients with X-linked adrenoleukodystrophy and in a mouse model of X-linked adrenoleukodystrophy, and its expression is reduced by antioxidants *in vivo*

Evidence for oxidative damage underlying neurodegeneration in X-linked adrenoleukodystrophy patient brains abounds (Gilg *et al.*, 2000; Powers, 2005; Fourcade *et al.*, 2008; Ferrer *et al.*, 2010; Galea *et al.*, 2012). We therefore assessed cyclophilin D expression in the brains of patients with cerebral adrenomyeloneuropathy. We found accumulation of cyclophilin D in the affected white matter areas (Fig. 8), although no significant differences in the non-demyelinated areas were found.

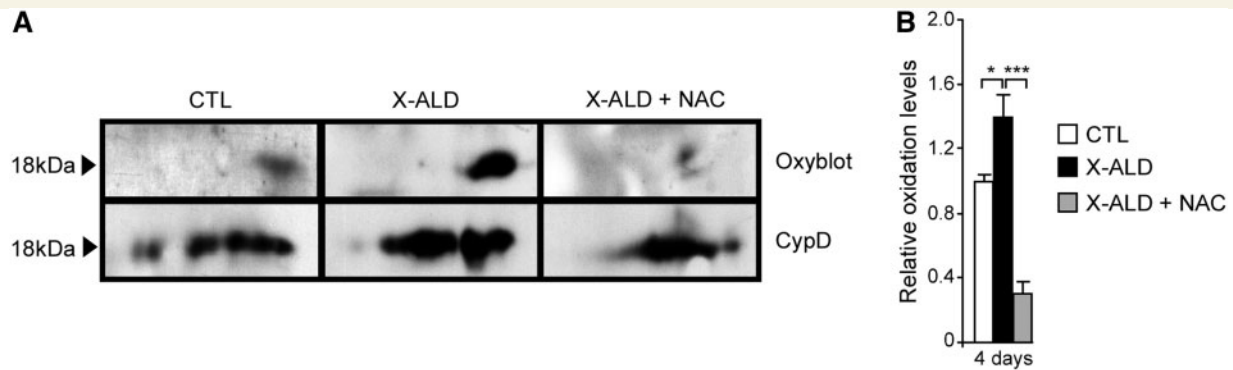
The mouse model of X-linked adrenoleukodystrophy presents long tract axonopathy in spinal cords, along with microgliosis and astrocytosis, accompanied by peripheral nerve conduction

impairment and motor disability ~20 months of age, thus allowing for a wide window for monitoring the pathogenic cascade and for therapeutic intervention (Pujol *et al.*, 2002, 2004). However, oxidative damage to proteins appears early, ~3 months of age (Fourcade *et al.*, 2008). No changes in cyclophilin D levels were observed at this early age in wild-type and *Abcd1*<sup>-</sup> mice spinal cord (Supplementary Fig. 2A). However, later in time, but before disease onset, at 8 months of age, we observed increased cyclophilin D levels when compared with wild-type littermates (Fig. 9A). In addition, cyclophilin D levels were unchanged in brain cortex and liver of 8-month-old mice (Supplementary Fig. 2B and C), suggesting that cyclophilin D expression induction is target-organ specific and precedes axonal degeneration.

We have previously shown that a combination of antioxidants, including *N*-acetylcysteine and  $\alpha$ -lipoic acid, reversed metabolic failure and axonal degeneration in an X-linked adrenoleukodystrophy mouse model (Galino *et al.*, 2011; Lopez-Erauskin *et al.*, 2011). To investigate whether these antioxidants were effective counteracting cyclophilin D overexpression, we treated 8-month-old *Abcd1*<sup>-</sup> mice with a combination of *N*-acetylcysteine and  $\alpha$ -lipoic acid for 4 months and found that the levels of cyclophilin D were normalized by antioxidants (Fig. 9B).

## Discussion

Neurodegenerative disorders are often linked to oxidative stress and mitochondrial dysfunction, including pathological mitochondrial permeability transition pore opening (Lin and Beal, 2006; Su *et al.*, 2009). In the present report, we have uncovered



**Figure 7** Antioxidant treatment halts cyclophilin D oxidation in X-linked adrenoleukodystrophy fibroblasts. Oxyblot and western blot with specific antibody against cyclophilin D were performed to compare control and X-linked adrenoleukodystrophy fibroblasts treated or not with 1 mM of *N*-acetylcysteine. Three independent experiments were performed for each condition. Representative blots are shown (**A**). Relative protein oxidation levels were quantified and expressed as a percentage of control and referred to cyclophilin D expression (**B**) ( $*P < 0.05$ ;  $***P < 0.001$ ;  $n = 3$ /genotype and condition). CTL = control; CypD = cyclophilin D; NAC = *N*-acetylcysteine; X-ALD = X-linked adrenoleukodystrophy.

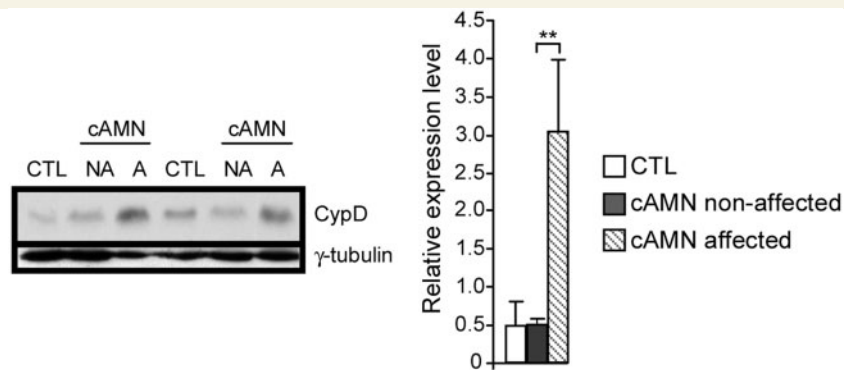
novel links between oxidative stress, mitochondrial depolarization, ATP loss and ensuing cell death in X-linked adrenoleukodystrophy. We found that enforcement of mitochondrial metabolism by culturing X-linked adrenoleukodystrophy fibroblasts in galactose was sufficient to promote cell death. Although control and X-linked adrenoleukodystrophy fibroblasts underwent oxidative stress under galactose conditions, only X-linked adrenoleukodystrophy fibroblasts displayed the loss of ATP and mitochondrial depolarization that preceded cell death, in an oxidative stress-dependent manner. These data suggest that X-linked adrenoleukodystrophy cells are particularly sensitive to oxidative stress, and that an excess of radical oxygen species production is responsible for the opening of the mitochondrial permeability transition pore and resulting cell death. We propose that high levels and/or increased oxidation of cyclophilin D mediate or contribute to this sensitivity. Moreover, we conclude that the oxidative stress that emerges from the accumulation of very long-chain fatty acids (Fourcade *et al.*, 2008; Baarine *et al.*, 2012a) triggers necrotic cell death in X-linked adrenoleukodystrophy fibroblasts. We show that this is associated with non-apoptotic chromatin condensation (absence of cells with totally condensed and/or fragmented nuclei), ATP drop and mitochondrial swelling, and it does not involve caspase activation. Along the same lines, and although formal proof for necrosis in neural tissue is lacking, we had formerly shown lack of caspase-3 activation in spinal cords of both *Abcd1* null and *Abcd1/Abcd2* null mice (Pujol *et al.*, 2004). Interestingly, very long-chain fatty acids, such as C24:0 or C26:0, also provoke necrotic cell death in cultured oligodendrocytes (Kahn *et al.*, 2011). These results offer the opportunity of future studies aiming to delve deeper into characterization of specific forms of necrosis, such as necroptosis. This is a form of cell death frequently associated with bioenergetic stress and which requires RIPK1 or RIPK3 proteins (Degterev *et al.*, 2008).

The vulnerability of X-linked adrenoleukodystrophy fibroblasts to mitochondria-dependent energy production invites the speculation that specific neural cell types, which largely depend on

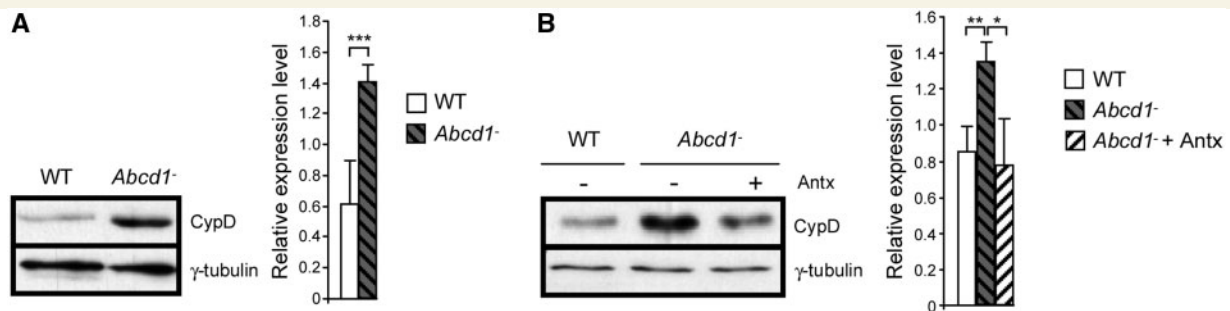
mitochondria for their energy supply, may be preferentially exposed and jeopardized *in vivo*. Indeed, axons have high metabolic demands and unusual length that renders them susceptible to oxidative damage, ischaemia or mitochondrial defects, as seen in multiple sclerosis (Lassmann, 2011) or amyotrophic lateral sclerosis (Shi *et al.*, 2010). It follows that malfunctioning mitochondria could be an important factor accounting for the long-tract axonopathy of spinal cords observed in patients with adrenomyeloneuropathy and the X-linked adrenoleukodystrophy mouse models.

Although the consequences of the mitochondrial permeability transition pore opening have been extensively studied in many laboratories, its exact molecular identity remains unknown. Knockout-based studies have confirmed that cyclophilin D plays an essential role regulating mitochondrial permeability transition pore opening and necrotic cell death, although cyclophilin D is neither a structural component of the pore nor a transmembrane protein (Baines *et al.*, 2005; Nakagawa *et al.*, 2005; Schinzel *et al.*, 2005). Cyclophilin D, located in the mitochondrial matrix, has been suggested as helping in the correct folding of proteins (Matouschek *et al.*, 1995). However, in response to pathological insults, such as calcium overload and/or oxidative stress, cyclophilin D is translocated to the inner mitochondrial membrane to induce mitochondrial permeability transition pore formation (Connern and Halestrap, 1994).

One major consequence of mitochondrial permeability transition pore opening is that the inner mitochondrial membrane no longer maintains a barrier to protons, which leads to dissipation of the proton motive force. The resulting uncoupling of oxidative phosphorylation not only prevents mitochondria from making ATP, and produces cytotoxic radical oxygen species, but also the proton-translocating ATP synthase goes into reverse, consuming ATP, due to futile cycles in an effort to restore the inner mitochondrial membrane potential, thereby further contributing to cellular damage (Halestrap, 2009; Zorov *et al.*, 2009). In this study, we have demonstrated that oxidative stress leads to energetic failure and necrotic cell death mediated by opening of mitochondrial



**Figure 8** Cyclophilin D is accumulated in affected white matter brain zones of cerebral adrenomyeloneuropathy patients. Cyclophilin D levels were analysed in control and in different areas of brains in patients with cerebral adrenomyeloneuropathy (\*\* $P < 0.01$ ;  $n = 4$ /genotype and condition). A = affected; cAMN = cerebral adrenomyeloneuropathy; CTL = control; CypD = cyclophilin D; NA = non-affected.



**Figure 9** Antioxidant treatment prevented cyclophilin D increase in *Abcd1*<sup>-/-</sup> mice spinal cord. Cyclophilin D expression levels were quantified in wild-type and *Abcd1*<sup>-/-</sup> mice spinal cord at 8 months (A) and at 12 months after an antioxidant cocktail treatment (B) (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ;  $n = 5$ /genotype and condition). Antx = antioxidants; CypD = cyclophilin D; WT = wild-type.

permeability transition pore, concomitant with cyclophilin D over-expression and oxidation, in fibroblasts of patients with X-linked adrenoleukodystrophy. Mitochondrial dysfunction characterized by ATP drop, inner mitochondrial membrane potential dissipation and cell death is prevented with both cyclosporin A (inhibitor of cyclophilin D) and the antioxidant *N*-acetylcysteine in this system. Taken together, these results show that (i) mitochondrial permeability transition pore opening is implicated in cell death and (ii) oxidative stress is the main inducer of mitochondrial permeability transition pore opening-induced cell death in X-linked adrenoleukodystrophy fibroblasts. Additionally, we have demonstrated that cyclophilin D is more markedly carbonylated in X-linked adrenoleukodystrophy fibroblasts, and that antioxidants reverse these oxidative modifications. Recently, cyclophilin D has been suggested as a redox sensor where oxidation would change its conformation, translocating it to the inner mitochondrial membrane and forming the pore (Linard *et al.*, 2009; Nguyen *et al.*, 2011). We therefore propose cyclophilin D as a key molecule in X-linked adrenoleukodystrophy physiopathology and a prime novel therapeutic target for intervention.

Given the positive correlation of cyclophilin D expression with mitochondrial permeability transition pore opening (Karlsson *et al.*,

2004; Baines *et al.*, 2005; Brown *et al.*, 2006; Du *et al.*, 2008), we predict that cells with increased expression of cyclophilin D may be primed for mitochondrial permeability transition pore formation and the ensuing consequences. Interestingly, we observed increased expression of cyclophilin D in X-linked adrenoleukodystrophy mouse model spinal cord and an induced cyclophilin D expression in control human fibroblasts after placing them on galactose or excess of C26:0, in an oxidative stress-dependent manner. Also, we found elevated cyclophilin D expression levels in affected zones in brains of patients with cerebral adrenomyeloneuropathy, in a similar manner to the increased cyclophilin D levels that were observed in affected brain regions of patients with Alzheimer's (Du *et al.*, 2008) and Huntington's disease (Shirendeb *et al.*, 2011). Moreover, higher levels of cyclophilin D have been found preceding axonal degeneration of the Wallerian type (Barrientos *et al.*, 2011).

Further, antioxidant treatment decreases cyclophilin D expression levels in both *in vitro* and *in vivo* models of X-linked adrenoleukodystrophy, which confirms that oxidative stress regulates cyclophilin D expression. In agreement with this, it has recently been reported that mitochondria-targeted antioxidants, such as MitoQ, and resveratrol significantly decrease cyclophilin D



expression levels in primary hippocampal neuron cultures from amyloid- $\beta$  precursor protein transgenic mice (Tg2576 line), protecting cells against amyloid- $\beta$  toxicity (Manczak *et al.*, 2010). Of note, we show here for the first time that chronic oral treatment with a combination of classical antioxidants is able to lower both cyclophilin D expression and oxidation levels in the CNS *in vivo*.

Our findings emphasize the importance of cyclophilin D as target for neurodegenerative diseases. Indeed, blockade of cyclophilin D by genetic abrogation or pharmacological inhibition protects mitochondria in many age-related chronic neurological disease models, such as Alzheimer's disease (Du *et al.*, 2008, 2011), Parkinson's disease (Gandhi *et al.*, 2009; Thomas *et al.*, 2011), amyotrophic lateral sclerosis (Karlsson *et al.*, 2004; Martin *et al.*, 2009; Martin, 2010) and multiple sclerosis (Forte *et al.*, 2007; Martin, 2010). The latter studies suggest that cyclophilin D-dependent mechanisms are critical in the neurodegenerative aspects of demyelinating and motor neuron diseases. Thus, molecules crossing blood–brain barrier able to target cyclophilin D expression or activity hold great promise in the treatment of cyclophilin D-dependent diseases of the nervous system, including X-linked adrenoleukodystrophy. As cyclosporin A and non-immunosuppressive derivatives show poor CNS penetration (Begley *et al.*, 1990), drug modification and use of barrier-permeable delivery vehicles may need to be taken into account, as well as drug targeting to mitochondria (Camara *et al.*, 2010).

In summary, the present study unveils cyclophilin D as a plausible effector preventing axonal degeneration in X-linked adrenoleukodystrophy and as a molecular target for antioxidant actions. This emphasizes the rationale for mitochondria-targeted antioxidant strategies in other neurodegenerative and non-neurodegenerative cyclophilin D-dependent disorders.

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## Supplementary material

Supplementary material is available at *Brain* online.

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