Conservation of targeting but divergence in function and quality control of peroxisomal ABC transporters: an analysis using cross-kingdom expression.

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Short title: Cross-kingdom targeting and function of peroxisomal ABC transporters

SYNOPSIS

ABC subfamily D transporters are found in all eukaryotic kingdoms and are known to play essential roles in mammals and plants; however their number, organisation and physiological contexts differ. Via cross-kingdom expression experiments, we explored the conservation of targeting, protein stability and function between mammalian and plant ABCD transporters. When expressed in tobacco epidermal cells, the mammalian ABCD proteins, adrenoleukodystrophy protein (ALDP), ALD related protein (ALDR) and PMP70 targeted faithfully to peroxisomes and P70R targeted to the ER, as in the native host. The Arabidopsis thaliana peroxin, AtPex19_1 interacted with human peroxisomal ABC transporters both in vivo and in vitro, providing an explanation for the fidelity of targeting. The fate of X-linked adrenoleukodystrophy disease-related mutants differed between fibroblasts and plant cells. In fibroblasts, levels of ALDP in some ‘protein absent’ mutants were increased by low-temperature culture, in some cases restoring function. In contrast, all mutant ALDP proteins examined were stable and correctly targeted in plant cells, regardless of their fate in fibroblasts. ALDR complemented the seed germination defect of the Arabidopsis cts-1 mutant which lacks the peroxisomal ABCD transporter, CTS, but neither ALDR nor ALDP was able to rescue the defect in fatty acid β-oxidation in establishing seedlings. Taken together, the data indicate that the mechanism for trafficking of peroxisomal membrane proteins is shared between plants and mammals, but suggest differences in the sensing and turnover of mutant ABC transporter proteins and differences in substrate specificity and/or function.

KEYWORDS: ABC transporter, Pex19, peroxisome, X-ALD, ALDP, PMP70, CTS

ABBREVIATIONS: ABC, ATP binding cassette; ALDP, adrenoleukodystrophy protein; ALDR, adrenoleukodystrophy related protein; CTS, comatose; FA-CoA, fatty acyl CoA;
NBD, nucleotide binding domain; PMP, peroxisomal membrane protein; PMP70, 70 kDa peroxisomal membrane protein; P70R, PMP70-related protein; TMD, transmembrane domain; VLCFA, very long chain fatty acid; X-ALD, X-linked Adrenoleukodystrophy.

INTRODUCTION

Peroxisomes are near-ubiquitous organelles with a wide range of functions in different eukaryotes. The β-oxidation of fatty acids is a function shared with mitochondria in mammals but exclusive to peroxisomes in plants and yeast. The importance of these organelles is underscored by the spectrum of human diseases associated with impaired peroxisomal function [1] and also by the severe, often embryo-lethal phenotypes observed for plant mutants lacking or defective in key peroxisome proteins [2-5]. The most common human peroxisomal disorder is X-linked Adrenoleukodystrophy (X-ALD), which is characterised biochemically by the accumulation of very long chain fatty acids (VLCFA) in plasma and tissues [6,7]. The primary genetic cause of this disease is mutation of the ABCD1 gene, which encodes an ATP Binding Cassette (ABC) transporter, Adrenoleukodystrophy protein (ALDP or ABCD1) [8]. ALDP belongs to subfamily D of the ABC protein superfamily, which has three further members in humans: Adrenoleukodystrophy-related protein (ALDR; ABCD2), 70 kDa peroxisomal membrane protein (PMP70; ABCD3) and the PMP70-related protein (P70R; ABCD4, also known as PMP69), encoded by ABCD2-4, respectively [9]. The human ABCD proteins are so-called “half-size” ABC transporters, containing one transmembrane domain (TMD) and one nucleotide binding domain (NBD), and thus must dimerise to form a functional transporter, which compromises 2 TMDs and 2 NBDs [10]. Although heterodimerisation between human ABCD proteins has been demonstrated in vitro and in vivo, expression patterns, protein purification, functional analysis and genetic considerations suggest that homodimerisation predominates in vivo and is sufficient for function (reviewed in [6,11]).

The ABCD subfamily is smaller in other organisms, with two members in baker’s yeast: Pxa1p and Pxa2p. Genetic evidence indicates that these proteins form a heterodimeric transporter required for import of long chain long chain fatty-acyl CoA esters (FA-CoAs) into the peroxisome [12-14]. Increased levels of VLCFA-CoA in X-ALD fibroblasts [15] and complementation of the yeast pxal pxa2Δ mutant by human ABCD1 [16] provided evidence that ALDP is also able to transport very long chain FA-CoA. Several lines of indirect evidence point to overlapping, but distinct substrate specificities of ALDR and ALDP. ALDR expression restores β-oxidation of C24:0 and C26:0 to pxal pxa2Δ cells, though not as efficiently as ALDP [17] and rescues β-oxidation activity and VLCFA levels in fibroblasts and Abcd1-/- knockout mice [6,11]. However, ALDR appears to play a specific role in the synthesis of the polyunsaturated VLCFA, docosahexaenoic acid (C22:6ω3), which requires chain-shortening of C24:6ω3 by β-oxidation [17-19]. Over-expression or pharmacological induction of PMP70 can also partially compensate for the absence of functional ALDP in X-ALD fibroblasts [20, 21] and silencing of ABCD3 in glial cells suggests a function in C26:0 metabolism [22]. However, PMP70 has also been suggested to play a specific role in the synthesis of bile acids [23]. The biochemical function of P70R is not known but the protein has recently been shown to be localised in the ER, rather than in peroxisomes [24].

Peroxisomal ABCD proteins encoded in plant genomes are full-size transporters, which are considered to represent “fused heterodimers”, exemplified by the Arabidopsis thaliana...
transporter, Comatose (CTS; also known as AtPXA1, PED3, ACN2 and AtABCD1) [25-28]. Extensive characterisation of cts null mutants has demonstrated key roles for CTS in seed germination, seedling establishment, root development, fertility and dark-induced senescence [25-27, 29-33]. Several of the phenotypes of cts mutants reflect their inability to mobilise storage triacylglycerol (TAG) and to turn over membrane lipids by β-oxidation [26, 27, 31, 32]. CTS complements the yeast pxal px2Δ mutant for growth on oleate and mediates β-oxidation of a range of saturated and unsaturated FA with chain lengths from C18 to C24, when expressed in yeast cells [34]. However, the functions of CTS also include acetate metabolism [28] and roles in the β-oxidation of ring-containing molecules, including 12-oxophytodienoic acid, the precursor of the plant hormone, jasmonic acid [35], the auxin analogue, 2,4-dichlorophenoxybutyric acid (2,4-DB) [27] and the natural auxin, indole butyric acid (IBA) [25]. Thus, in contrast to mammals, which have several peroxisomal ABC transporters with distinct roles but overlapping specificity, plant peroxisomes contain a broad specificity transporter involved in signalling and metabolism.

Peroxisomal ABC transporters, in common with other peroxisomal membrane proteins (PMPs) utilise the receptor/chaperone Pex19 for targeting and membrane insertion [36]. Recent structural data provide a plausible mechanism for targeting signal recognition, chaperone function and docking to Pex3 at the peroxisome membrane [37, 38]. Although most studies have focused on PMPs in humans and yeast, Pex19 and Pex3 appear to be conserved between animals, plants and fungi [36]. However, Pex19 proteins are rather divergent at the amino acid sequence level, and unlike animals and fungi, many plant species contain two Pex19 forms, with apparently distinct functions, as judged by RNAi studies [5]. Therefore we were curious to determine to what extent peroxisomal targeting is functionally homologous between mammals and plants and also whether ABCD functions are shared between different major taxonomic groups.

In this study, we provide evidence that Pex19 function is largely conserved between the two kingdoms and that mammalian PMPs are correctly targeted in plant cells. We found that selected “unstable” X-ALD mutants can be rescued by low-temperature culture of fibroblasts but that a wide range of missense mutants produce detectable ALDP in peroxisomes when expressed in plants, indicating that plant and mammalian cells exhibit significant differences in the fate of mutant PMPs. Furthermore, we demonstrate that the human peroxisomal ABC transporters, ALDP and ALDR, although correctly targeted when expressed in Arabidopsis, are mostly unable to substitute for the endogenous CTS transporter.

EXPERIMENTAL

Construction of plant expression plasmids
Sequence-verified IMAGE consortium collection cDNA clones [39] for subfamily D ABC transporters were obtained from GeneService (Cambridge, UK): human ABCD1 (accession no. BC015541), mouse ABCD3 (accession no. BC054446), human ABCD4 (accession no. BC012815) or Open Biosystems (Huntsville, AL, USA): human ABCD2 (accession no. BC104901). ORFs were amplified by PCR using the primer pairs shown in Table S1 to incorporate attB sites and recombined into the entry vector, pDONR207, and then recombined into the plant binary destination vector, En35S-Cassette b-eYFP-Nos::pCAMBIA 1300 [40], using Gateway technology (Invitrogen, Paisley, UK), to generate ABCD-YFP fusions for expression in plants. All constructs were sequenced to establish authenticity.
For nuclear mis-localisation experiments, the AtPEX19_1 ORF was amplified without its native start codon from AtPex19_1/pET28b [41], using primer pair AtPex19SalIFor/AtPex19SacIIRev and inserted into pcR4blunt (Invitrogen), to create pcR4blunt-AtPex19. A 447 bp fragment containing a nuclear localisation signal (NLS) was amplified from histone 2B (H2B) cDNA [42], using primer pair H2BSallFor/H2BXhoIRev and inserted into pcR4blunt (Invitrogen) to create pcR4blunt-H2B. A Sal I-H2B-XhoI fragment was excised from pcR4blunt-H2B and ligated to Sal I-digested pcR4blunt-AtPex19-1, to create pcR4blunt-H2B-AtPex19-1. The H2B-AtPex19-1 fragment was amplified with primer pair H2BAtpex19attB1/H2BAtpex19attB2 to add attB sites for Gateway cloning and recombined into pDONR207-H2B-AtPex19-1 to create an entry clone. Following recombination into a Gateway-adapted pCAMBIA1300 vector to create the binary construct pCAMBIA1300-H2B-AtPex19-1, the final construct was sequenced.

Site-directed mutagenesis was carried out using the QuikChange II kit (Stratagene, La Jolla, CA), according to manufacturer’s instructions. Primer sequences are given in Table S1. Constructs were sequenced to verify the presence of the mutation and to confirm that no unwanted mutations had been introduced.

**Expression and purification of GST-Pex19 fusion proteins.**

A human Pex19 (HsPex19) IMAGE consortium clone, accession number BC000496 [39] was obtained from GeneService (Cambridge, UK). AtPex19_1 [41] and HsPex19 were amplified with primer pairs FT119/FT120 and FT123/124, respectively, restricted with Bam HI/Not I and cloned in the corresponding sites of pGEX-4T-3 (GE Healthcare, Ltd, UK). Expression of GST fusion proteins in *E. coli* strain BL21 (DE3) was induced by IPTG (0.1 mM) for 3 h. Cells were harvested by centrifugation at 6,000 rpm for 15 min at 4 °C, resuspended in 10 ml of PBS containing 1 mM PMSF and 10 mM benzamidine and lysed by sonication. Triton X-100 and DTT were added to the lysate to give final concentrations of 1 % (v/v) and 1 mM, respectively. Following 30 min incubation at 4 °C, the lysate was clarified at 7,000 rpm for 10 min at 4°C. Fusion proteins were subjected to affinity purification using glutathione magnetic beads (Thermo Scientific, Surrey, UK), according to the manufacturers’ instructions.

**In vitro transcription/translation and pull-down assays**

ALDR and PMP70 were amplified with primer pairs FT125/FT126 and FT127/128, respectively, restricted with Bam HI/Xba I and cloned into the corresponding sites of pcDNA3.1/neo(+) (Invitrogen). ALDP/pcDNA3.1 is described in [43]. The proteins were transcribed *in vitro* from the T7-promotor, translated and labelled with FluoroTect GreenLys tRNA using the TNT Coupled Reticulocyte Lysate System (Promega, UK) following standard protocols. Purified GST fusion proteins were bound to glutathione-Sepharose beads (Thermo Scientific) in Tris-buffered saline (TBS) for 1 h at 4 °C and washed three times in phosphate buffered saline (PBS). For the *in vitro* interaction assay, 2 mg GST or GST-PEX19 protein was bound to 300 μl bead suspension and incubated were incubated with 50 μl of *in vitro* translation reaction product in 250 μl binding buffer [100 mM NaCl, 50 mM potassium phosphate pH 7.4, 1 mM MgCl2, 10 % (v/v) glycerol, 0.1 % (v/v) Tween 20, 1.5 % (w/v) BSA] for 2 h at 4°C. Samples were pelleted and washed four times in 1 ml binding buffer without BSA. Pellets were resuspended in SDS-sample buffer and boiled for 3 min before being analyzed by SDS–PAGE. The gels were scanned with a Typhoon® 8600 scanner (GE Healthcare, UK), at 532 nm excitation. To confirm equal loading, the gels were silver-stained after each pull-down experiment.
Growth and transformation of Arabidopsis and tobacco plants

Tobacco (*Nicotiana tabacum*) plants were grown and transiently transformed by Agro-infiltration as described in [40, 44]. Arabidopsis (*Arabidopsis thaliana* accession Landsberg erecta; Ler) plants were transformed with ALDP-YFP/pCAMBIA 1300 and ALDR-YFP/pCAMBIA 1300 by the floral dip method [45], as were *cts-1* plants and plants heterozygous for *cts-1*. The *cts-1* mutant is described in [26].

Microscopy

Confocal imaging was performed using a Zeiss inverted LSM510 laser scanning confocal microscope with an argon laser, a blue diode laser and a helium neon laser and 40x objective. For imaging co-expression of CFP and eYFP, excitation lines were 405 nm for CFP and 514 nm for eYFP. For imaging co-expression of GFP and eYFP, excitation lines 458 nm (GFP) and 514 nm (eYFP) were used alternatively with line switching in the multitracking mode of the microscope. For imaging co-expression of DAPI and eYFP, excitation lines 405 nm and 514 nm were used. Fluorescence was detected using a 505-530-nm band-pass filter for green fluorescent protein (GFP), a 530-600 nm band pass filter for enhanced yellow fluorescent protein (eYFP), 420-480 for CFP and DAPI. The pinhole was usually set to give an optical slice between 1-2.5 μm and controls were performed to prevent bleed through of fluorescence and cross-talk. Post acquisition image processing was performed using the LSM 5 browser software (Zeiss) and Adobe Photoshop (San Jose, CA) elements.

Phenotypic characterisation of plants

Germination, establishment, hypocotyl and root growth assays were performed as described in [46], unless otherwise stated. Fatty acid methyl esters were analysed as described in [47], using heneicosanoic acid, C21:0 (Nuchek-prep, Inc., Elysian, MN, USA), as an internal standard.

Fibroblast methods

Human skin fibroblasts were obtained from X-ALD patients (aged 23-63) through the Neurology Outpatient Clinic of the Academic Medical Centre. Written informed consent was received from each patient. X-ALD diagnosis was confirmed by VLCFA and *ABCD1* mutation analysis. Control fibroblasts were from male anonymous volunteers (aged 20-50). Cells were grown in HAMF10 supplemented with 10% foetal calf serum, 25 mM HEPES, 100 U/mL penicillin, 100 U/mL streptomycin and 2 mM glutamine. Cell lines were cultured in a humidified atmosphere of 5% CO₂ at 37°C. For some experiments, fibroblasts were cultured at 30°C for the time indicated, after an overnight incubation at 37°C.

Immunoblotting

Cells were harvested by trypsinisation and homogenised by sonication in PBS containing protease inhibitors. Homogenates were diluted in sample buffer before 50 μg of total protein was loaded on a 10% SDS polyacrylamide gel. Proteins were transferred to nitrocellulose by semi-dry blotting, blocked with BSA and probed with the primary monoclonal antibody against human ALDP (ALD-1D6-As, Euromedex). IRDYE 800CW (LI-COR Biosciences) goat anti-mouse IgG was used as secondary antibody. Visualization of the signal was done with the Odyssey infrared imaging system (LI-COR Biosciences). For quantification of ALDP expression levels, a calibration curve was used on each blot. The calibration curve was made by mixing different amounts of control sample (ranging from 100 to 0%) with increasing amounts of protein from an ALDP frameshift (p.Arg113fs) sample (ranging from 0% to 100%).
Quantitative RT-PCR of \textit{ABCD1} mRNA levels

\textit{ABCD1} mRNA expression levels were determined as described [48]. mRNA was isolated from cells growing in log-phase and \textit{PPIB} (cyclophilin B) was used as a housekeeping gene. Primer sequences are given in Table S1.

Peroxisomal \(\beta\)-oxidation and analysis of VLCFA in fibroblasts

Peroxisomal \(\beta\)-oxidation activity in whole cells using 15 \(\mu\)M \(\mathrm{D}_{3}\)-docosanoic acid (\(\mathrm{D}_{3}\)-C22:0; CDN Isotopes, Québec, Canada) was determined following our previously developed method [49]. Total cellular fatty acids were analyzed using the electrospray ionization mass spectrometry method described previously [50].

Supplementary methods

**Quantification of \textit{ABI5} transcripts**

Seeds were plated on 0.5 X MS medium, containing 1\% (w/v) sucrose and dark-chilled for 2 d at 4 \(^\circ\)C. After 48 h, RNA was extracted using the Qiagen RNeasy kit according to the manufacturers’ instructions. First-strand cDNA synthesis was carried out using SuperScript III reverse transcriptase and oligo dT (Invitrogen), according to the manufacturers’ instructions. RT-PCR was carried out using the following primer pairs: \textit{ABI5for/ABI5rev} and \textit{18SrRNAfor/18SrRNArev}. Quantitative PCR was carried out using the 7900HT Fast Real-Time PCR system (ABI) containing power SYBR Green for cDNA analysis in a final reaction volume of 10 \(\mu\)l. An alternative housekeeping primer set (GAPDHfor/GAPDHrev) gave comparable results.

Subcellular localisation of \textit{ALDP-YFP and ALDR-YFP} in Arabidopsis

Transgenic lines expressing \textit{ALDP-YFP/pCAMBIA 1300} were crossed to Arabidopsis plants stably expressing \textit{CFP-SKL} [40], to confirm peroxisomal localisation by confocal imaging as described in \textit{Experimental}. Seeds from the lines \textit{PEX10-YFP} [40], \textit{Ler/ALDR-YFP} and \textit{cts-I/ALDR-YFP} were sterilised and sown on 0.5 x MS medium, stratified for 2 d at 4 \(^\circ\)C and transferred to a growth chamber (20 \(^\circ\) C; 16 h light) for 3 d. Seedlings were removed from the plates and stained with BODIPY 8 [51] as follows: a 3 mM solution of BODIPY 8 in ethanol was diluted 1:1000 in water immediately before use and seedlings were immersed in the solution for a minimum of 15 min before viewing on an LSM510 meta confocal microscope. The remaining \textit{cts-I/ALDR-YFP} seedlings were returned to the growth chamber for 2 d then transferred to 0.5 x MS containing 1\% (w/v) sucrose and grown for a further 5 d to allow establishment, stained with BODIPY 8 and imaged as before. Excitation lines were 488 nm for eYFP and fluorescence was detected using a 505-530-nm band-pass. Excitation of BODIPY 8 was performed with a 633 nm laser and fluorescence was detected at 650 nm.

RESULTS

Mammalian ABCD proteins are correctly targeted \textit{in planta}

In order to test the targeting of mammalian ABCD transporters in plants, the proteins were tagged at the C-terminus with enhanced yellow fluorescent protein (eYFP) and their subcellular localisation was investigated by confocal microscopy following transfection of tobacco leaves by agroinfiltration. YFP fusions of \textit{ALDP}, \textit{ALDR} and \textit{PMP70} were transiently expressed in the epidermal cells of plants stably expressing the peroxisomal marker, cyan fluorescent protein (CFP)-SKL (Fig. 1A-C). Peroxisomes were observed as discrete spots in the CFP channel, in the majority of cells examined. The majority of
peroxisomes were found at the cell margins, owing to the presence of the large central vacuole, characteristic of epidermal cells. Typically, agroinfiltration does not result in all of the cells being transfected (not shown); however, in cells which expressed YFP, punctate structures were observed in the YFP channel. These coincided with the peroxisomal CFP signal, as seen in the merged images. At higher magnifications (Fig. 1A-C, lower panels), the YFP signal exhibited a ring-like appearance, characteristic of peroxisomal membrane proteins, with the matrix-located CFP-SKL inside the peroxisome structure (e.g. [40]). These images indicate that ALDP, ALDR and PMP70 are all targeted to the peroxisomal membrane in plant cells, consistent with their localisation in mammalian cells. In contrast, examination of leaf sections transfected with P70R-YFP did not reveal punctate structures but rather a reticulate YFP pattern. Therefore P70R-YFP fusions were transiently expressed in leaves of tobacco plants stably expressing the ER reporter GFP-HDEL (Fig.1D). In cells where both reporters were expressed, the GFP and YFP co-localised, suggesting that P70R-YFP is located in ER, in agreement with its localisation in mammalian cells (Kashiwayama et al., 2009).

Mammalian peroxisomal ABC transporters interact with plant Pex19 in vitro and in vivo

The targeting of ALDP, ALDR and PMP70 to peroxisomes when expressed in planta suggests conservation of peroxisomal membrane protein targeting machinery between the animal and plant kingdoms and is consistent with studies demonstrating that PMP targeting is conserved between yeast, trypanosomes and humans [52-54]. To investigate this further, we used two independent assays to determine whether the mammalian ABC proteins interact with the receptor/chaperone protein, Arabidopsis Pex19_1 (AtPex19_1) in vitro and in vivo. In pull-down assays, in vitro translated peroxisomal ABC transporters bound to glutathione-S-transferase (GST)-AtPex19 fusions immobilised on glutathione beads (Fig. 2). In the negative controls (GST only), a small amount of binding was observed, probably due to the hydrophobic nature of the membrane proteins, but this was modest, compared to the binding observed with GST-AtPex19_1. Interactions with Pex19 in vivo were tested using a nuclear mis-localisation assay [55]. AtPex19_1 and HsPex19 were fused to the nuclear localisation signal (NLS) of Arabidopsis histone 2B, [42]. Tobacco plants stably expressing CFP-SKL were co-transfected with ABCD-YFP fusions and NLS-Pex19 and examined by confocal microscopy (Fig. 3). The transfection efficiency was much lower for two genes than for one (data not shown): in some cells, the YFP signal coincided with CFP-SKL in punctate structures, indicating peroxisomal localisation of ALDP and ALDR and suggesting that NLS-Pex19 was not expressed. In some cells, no YFP signal was observed and peroxisomes were observed as blue fluorescent punctate structures. However, in a small number of cells, punctate structures were absent from the CFP channel and the YFP signal was located in large, roughly spherical structures, of which there was one per cell. These structures stained with DAPI, indicating that they are nuclei (Fig. S1). The number of cells containing YFP-positive nuclei varied between experiments (examples with a higher level of co-transfection are shown in Fig. S1, panels 1-P). Using this assay, we observed interactions between HsPex19 and ALDP (Fig. 3A-D; positive control) and NLS-AtPex19_1 and both ALDP and ALDR (Fig. 3E-L), but were not able to demonstrate AtPex19-1-dependent mis-localisation of PMP70, possibly because PMP70 was expressed much less efficiently in tobacco epidermis than ALDP and ALDR (data not shown). Alternatively this finding might have resulted from preferential interaction of PMP70 with endogenous tobacco Pex19. Both tobacco and Arabidopsis have two Pex19 genes; it is
possible that the different Pex19 forms exhibit different affinities towards different PMPs. This remains to be tested in future studies. The loss of punctuate structures in the CFP channel is consistent with the interaction of NLS-AtPex19_1 (and also NLS-HsPex19) with endogenous peroxins, disrupting peroxisome biogenesis by drawing Pex19-binding proteins into the nucleus. This suggests that key peroxins are being turned over in tobacco epidermal cells during the time window of the experiment (4-5 days).

**Temperature rescue of X-ALD missense mutants**

Although ALDP is an integral peroxisome membrane protein in wild type cells, in X-ALD patient cells, the majority of cases with missense mutations result in the absence or very marked reduction of ALDP from the membrane [56]. About 60 % of X-ALD ABCD1 mutations are missense mutations, 65 % of which result in no detectable ALDP, based on immunofluorescence (IF), indicating that they affect protein folding and stability. We developed a method based on protein blot analysis for quantification of low amounts of ALDP (Fig. S2A) and reinvestigated these findings. Immunoblot analysis revealed that residual ALDP is present in all missense cell lines analysed, though sometimes below the level of detection by IF (Table 1; Fig. S2B). Protein was not present in the control line, which contains a frameshift mutation (p.Arg113fs). ABCD1 RNA levels did not correlate well with levels of ALDP, suggesting that protein abundance is subject to post-transcriptional controls, consistent with the notion that missense X-ALD mutations affect protein folding and stability (Fig. S2B,C). A simple method to identify folding mutations is to lower the tissue culture temperature from 37°C to 30°C, allowing proteins more time to fold correctly and thus pass the quality control system. Therefore, X-ALD patient fibroblasts were cultured at 30°C, and the ALDP levels measured by quantitative immunoblotting. ALDP was not increased significantly in response to low temperature in 10 WT control lines tested (Fig. S3A), however, increased expression levels of ALDP were found in several of the X-ALD fibroblasts investigated, with the exception of p.Ser149Asn, Asp194His, p.Leu220Pro p.Arg389His, p.Ser606Leu and p.His667Asp which did not exhibit a statistically significant increase (Fig. 4A,B). The effect of reduced temperature on ABCD1 transcript abundance was variable, with no clear correlation to the effect on protein abundance (Fig. S3B).

We then examined whether ALDP function was rescued in selected cell lines which exhibited an increase in protein upon low-temperature culture. ALDP was increased from 2-4 % to ca. 20 % of WT levels in lines bearing the mutations p.Glu609Gly, p.Ala616Thr and p.Arg660Trp, from 1 to 10 % in p.Glu609Lys and p.Arg554His cells and from 45 to 75 % in the p.Asp194His line (Fig. 4A). VLCFA β-oxidation was measured in cells that were cultured at 30°C for 72 h, but in only one case (p.Ala616Thr) was the capacity to degrade VLCFA restored to near-control levels (Fig. 4C). However after four weeks’ culture at 30°C, VLCFA levels were partially corrected in p.Arg660Trp, p.Arg554His and p.Ala616Thr fibroblasts. The apparent delay in alteration of VLCFA levels reflects slow turnover of VLCFA in fibroblasts, as demonstrated previously in a study in which the pharmacological agent 4-phenylbutyrate was used to correct β-oxidation and VLCFA levels in X-ALD cells [57]. Lines bearing the p.Glu609Gly and p.Asp194His mutations did not show any correction of the C26:0/C22:0 ratio (Fig. 4D). Lack of functional rescue may result if the missense mutation has a negative effect on ALDP transport activity or alternatively if the increased protein level upon low-temperature cultivation is not accompanied by correct targeting of ALDP.

**X-ALD mutant proteins are expressed at the peroxisome in plant cells**
Although we and others have shown that missense X-ALD mutations result in ALDP instability in mammalian cells (Fig. 4; [56, 58]), in a previous study, we noticed that cts mutants which have “protein-absent”, disease-associated equivalents in human ABCD1 were expressed as stable, correctly targeted proteins in Arabidopsis [46]. Therefore we tested whether ALDP variants bearing missense mutations which result in protein mis-targeting or instability in mammalian cells would behave similarly when expressed in plant cells. In addition to the temperature-sensitive mutants identified above, a range of X-ALD mutants with different intracellular fates was selected (Table 2; [58]), with the goal of comparing the capacity of plant and mammalian cells for sensing and turnover of defective peroxisomal membrane proteins. These included relatively common mutants which are unstable in fibroblasts but which can be rescued by the application of proteasome inhibitors (p.Ser606Leu, p.Arg617His, p.His667Asp), the p.Arg104Cys mutant in which degradation of ALDP cannot be prevented by proteasome inhibitors and the p.Tyr174Cys mutant which is mis-targeted in fibroblasts [58]. These mutations were introduced into the ALDP-YFP construct and transiently expressed in tobacco leaves which were stably transformed with CFP-SKL. In each case, the mutant proteins were detected in peroxisomes of tobacco epidermal cells, regardless of their intracellular fate in mammalian cells (Fig. 5 and Fig. S4).

It is not possible to estimate protein turnover from transient transfection experiments, however, it is clear that the X-ALD proteins were targeted to the peroxisome in plant cells and that at least a proportion of the protein escaped proteasomal degradation, suggestive of increased stability. This points to differences in the sensing and turnover of defective PMPs in plants and animal cells.

We next considered whether the mutant forms of ALDP were more stable owing to the lower temperature at which plants are typically cultivated (ca. 22-25 ºC), compared to mammalian cells cultured at 37 ºC, by comparing the fates of X-ALD mutants which respond differently to low temperature rescue. ALDP proteins bearing p.Arg554His, p.Glu609Gly, p.Ala616Thr and p.Arg660Trp mutations are undetectable by immunofluorescence analysis of fibroblasts, but trace amounts are present, as revealed by immunoblotting (Table 1) and cultivation of fibroblasts at low temperature (30 ºC) leads to an increase in ALDP protein (Fig. 4A). In contrast, the ALDP level in p.His667Asp fibroblasts is not rescued by low temperature and X-ALD mutant p.Ser606Leu expresses IF-detectable ALDP at 37 ºC, with little increase upon low-temperature cultivation (Fig. 4A). Upon expression in tobacco epidermal cells, ALDP-YFP was observed in peroxisomes for each of these three classes of mutant, as judged by co-localisation with CFP-SKL (Fig. 5 and Fig. S4), suggesting that increased apparent stability of mutant ALDP in plant cells is not solely due to improved folding at lower temperature.

Differential ability of ALDP-YFP and ALDR-YFP to complement cts-1

Since ALDP and ALDR were correctly targeted when transiently expressed in tobacco leaves, we investigated whether the human ABCD1 and ABCD2 cDNAs could complement one or more of the different phenotypes of the Arabidopsis cts-1 mutant. cts-1 seeds are unable to germinate and complete seedling establishment but it is not clear whether the requirement of CTS for germination is related to the provision of energy and carbon skeletons from storage lipid catabolism, or due to the metabolism of a signal molecule by β-oxidation [29]. Therefore, two strategies were adopted to obtain plants homozygous for cts-1 which stably expressed ABCD-YFP fusions. Firstly, homozygous cts-1 plants were transformed directly and T1 seed were screened for the ability to germinate on 0.5 X MS medium plus antibiotic selection in the absence of exogenous sugars and mechanical disruption of the seed coat, both of which are required for the germination of cts seeds [26, 29]. Screening seeds from two independent transformations with 35S::ALDP-YFP or 35S::ALDR-YFP yielded no
transformants, suggesting either that these constructs did not complement the germination phenotype of *cts-1* and/or that they did not confer the ability to establish in the absence of exogenous sucrose. Thereafter, therefore, heterozygous plants (CTS/*cts-1*) were transformed with ALDP-YFP and T1 plants were identified by antibiotic selection and the genotypes confirmed by PCR (data not shown). These plants were allowed to self-fertilise and set seed; homozygous lines were obtained by inducing seeds to germinate by disrupting the seed coat and cultivating on medium containing sucrose. For ALDR-YFP, T1 transformants in the *cts-1* background were obtained by screening on medium containing antibiotics and sucrose.

The germination kinetics of homozygous lines expressing ALDP-YFP or ALDR-YFP in the *cts-1* background were compared with those of the corresponding wild type accession, *Ler* and the untransformed mutant, *cts-1*. Seeds were placed on 0.5 x MS medium, dark-chilled for 2 d and then transferred to a growth cabinet for 7 d. After 7 d, >75% of *Ler* seeds had germinated, but no germination was observed for *cts-1* seeds or *cts-1* lines expressing ALDP-YFP. In contrast to ALDP, >60% of *cts-1* seeds expressing ALDR-YFP germinated after 7 d (Fig. 6A; Fig. S5A). Expression of ALDR-YFP also repressed transcript levels of *ABI5*, a gene which has been proposed to inhibit germination in *cts* seeds [33] (Fig. S5B). However, development did not proceed further in the absence of sucrose, indicating that ALDR-YFP does not rescue the seedling establishment phenotype of *cts-1* (Fig. 6A).

To examine seedling establishment independently of germination, seeds expressing ALDP-YFP were placed on filters overlaid on 0.5 X MS medium containing sucrose and the seed coats gently disrupted with a needle. After 2 d dark chilling, plates were transferred to a growth chamber for 24 h to permit germination. Seeds were then transferred to medium lacking sucrose and returned to the growth chamber for a further 4-5 d. A duplicate plate was maintained on sucrose medium. After 5 days, *Ler* seedlings had established (as judged by the presence of green cotyledons) on media with and without sucrose, but the *cts-1* mutant and *cts-1* lines expressing ALDP-YFP did not establish on media lacking sucrose (Fig. 6B). All genotypes were able to establish in the presence of 0.5% sucrose (Fig. 6C), indicating that there were no deleterious effects associated with either transgene. To confirm correct targeting of ALDP, lines expressing ALDP-YFP were crossed to an Arabidopsis line stably expressing CFP-SKL [40] and the progeny examined by confocal microscopy. Punctate structures were observed in the YFP channel in hypocotyl cells; these coincided with peroxisomes visualised by CFP-SKL, indicating that ALDP-YFP is located in peroxisomes in Arabidopsis as well as in tobacco (Fig. S6A). ALDP-GFP was previously shown to be functional in fibroblasts [58], thus ruling out a negative effect of fusion to a fluorescent protein. The subcellular localisation of ALDR-YFP was tested by counterstaining with BODIPY 8, a YFP-compatible member of a series of BODIPY probes which target peroxisomes in plant cells [51]. The specificity of BODIPY 8 was confirmed using Arabidopsis plants stably expressing a YFP fusion of the peroxisomal protein, AtPex10 [40]. Co-localisation of BODIPY 8 with peroxisomes was observed, although not all organelles which stained with the probe were observed in the YFP channels, since the YFP bleached relatively rapidly and the signal was less intense than that of the dye (Fig. S6B). Co-localisation of the BODIPY signal and YFP in *Ler* and *cts-1* plants expressing ALDR-YFP demonstrated peroxisomal targeting of this fusion protein in Arabidopsis (Fig. S6C,D).

The inability of *cts-1* lines expressing ALDP-YFP or ALDR-YFP to establish in the absence of exogenous sucrose suggests that they are unable to mobilise seed storage TAG which is required for growth before the seedlings become fully photosynthetic. Therefore the fatty acid contents of seedlings were determined by gas chromatographic analysis of fatty acid

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methyl esters. As shown previously [26], cts-1 seedlings retain TAG-derived fatty acids by comparison to WT, as judged by the increased level of eicosenoic acid (20:1), which is considered to be a marker for TAG in Arabidopsis [59] (Fig. 7). The cts-1 mutant also exhibited a marked retention of C18 fatty acids and the VLCFA species, 20:0, 20:2 and 22:1. The fatty acid profiles of cts-1/ALDP-YFP and /ALDR-YFP seedlings were qualitatively and quantitatively similar to those of cts-1, confirming the inability of these transgenic lines to mobilise storage oil for the provision of carbon skeletons and energy during seedling establishment.

In WT plants, CTS is required for import of the pro-auxins, 2,4-dichlorophenoxybutyric acid (2,4-DB) and indole butyric acid (IBA) into the peroxisome where they are converted to bioactive auxins, 2,4-dichloroacetic acid (2,4-D) and indole acetic acid (IAA) respectively, by β-oxidation. cts mutants are unable to metabolise 2,4-DB and IBA and are therefore resistant to the growth inhibiting properties of these compounds [25, 27]. The ability of ALDP-YFP and ALDR-YFP to complement the 2,4-DB resistance phenotype of cts-1 was tested: seeds were induced to germinate as described above and then transferred to plates containing 1 μM 2,4-DB or 30 μM IBA. Neither ALDP-YFP nor ALDR-YFP restored WT sensitivity to these compounds (Fig. 6D,E), indicating that the human peroxisomal ABC proteins were unable to mediate peroxisomal import of pro-auxins, in the transgenic lines analysed.

DISCUSSION

Peroxisomal ABC transporters are found in all eukaryotic kingdoms, but their domain organisation and the physiological context in which they operate varies. However, all have profound effects on the physiology of the organism when mutated. By testing the ability of ABCD family members to be correctly targeted and to complement phenotypes in a heterologous host [16, 17, 45, and this paper], we can begin to discern conserved and specialised functions.

In this paper, we show that ABCD family targeting is conserved between the plant and animal kingdom, but protein quality control differs. Several studies have reported that various ALDP missense mutants do not produce protein in human fibroblasts, however we demonstrated that protein is present at low levels and protein levels of a subset of mutants can be increased by low-temperature culture. In some cases, this led to partial recovery of function. The potential utility of these findings clearly depends on the effect of a given mutation on ALDP function, but this opens up the possibility for therapeutic intervention aimed at stabilising and promoting the correct targeting of selected missense mutations using chemical chaperones. In plants however, all the X-ALD mutants were correctly targeted, regardless of their fate in fibroblasts. The mechanistic basis for this finding remains to be explored, however, it may have important implications for expression of heterologous membrane proteins in plants.

ALDP could not complement the germination or establishment phenotypes of cts-1 but ALDR could complement the germination phenotype. The failure to complement establishment is not surprising, given the reported substrate specificity of ALDP and ALDR [16, 17]. This separation of physiological function provides additional evidence to support the assertion that the processes of germination and establishment are biochemically distinct [29]. Conversely, the ability of CTS to support beta-oxidation of a wide range of fatty acids in
yeast points to a broad substrate specificity [34], whereas the mammalian ABCDs have much more restricted substrates (this study and [16, 17]).

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr Gareth Howell and Dr Laura-Anne Brown for advice and assistance with the microscopy and Dr Fred Beaudoin for guidance with fatty acid analysis of seedlings. We thank Barbara Johnson for maintenance of tobacco lines, Professor Johannes Berger for ALDP/pcDNA3.1, Stephen Parsons for ALDP/pDONR207, Dr. Jurgen Deneke for GFP-HDEL seeds and Drs Patrick Steel and Andrei Smertenko for generously providing BODIPY 8.

FUNDING

This research was supported by BBSRC grants BB/F007108/1 to FLT and BB/F007299/1 to AB/SAB and a grant from the Netherlands Organization for Scientific Research (VIDI-grant No. 91786328) to SK and the EU grant (FP7 LEUKOTREAT 241622) to SK. Rothamsted Research receives grant-aided support from the BBSRC of the UK.

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40. Sparkes, I.A., Hawes, C. and Baker, A. (2005) AtPEX2 and AtPEX10 are targeted to peroxisomes independently of known endoplasmic reticulum trafficking routes. Plant Physiol. 139, 690-700


**FIGURE LEGENDS**

**Figure 1. Targeting of mammalian ABCD-YFP fusions in plant cells**
(A-C) Tobacco plants stably expressing CFP-SKL were transiently transfected with constructs containing ABCD-YFP fusions driven by the cauliflower mosaic virus 35S promoter. Leaf epidermal cells were imaged using confocal microscopy; from left to right: CFP, YFP, bright field and merge. A, ALDP; B, ALDR; C, PMP70. Panel D: tobacco plants stably expressing GFP-HDEL were transiently transfected with 35S::ABCD4(P70R/PMP69)-YFP. Left to right: GFP, YFP, bright field and merge. Scale bars 10 μm. Images are representative of results from at least three independent experiments.

**Figure 2. Interaction of mammalian ABCD proteins with Arabidopsis Pex19 in vitro**
AtPex19_1 was expressed as a GST fusion protein in *E.coli*. Purified GST-AtPex19-1 (or GST only, negative control) was immobilised on glutathione beads and incubated with *in vitro* translated peroxisomal ABC transporters labelled with BODIPY-lysine. Interacting proteins were eluted with SDS-PAGE buffer and separated in a 12 % acrylamide denaturing gel. Labelled proteins were visualised by fluorography. 20 % input, 20 % of the ABCD proteins used in the pull-down experiment; GST, pull-down with GST alone; GST-AtPex19, pull-down with GST-AtPex19_1. Figures at the left hand side of the panel indicate the position of molecular weight markers (kDa). Results are representative of three independent experiments.

**Figure 3. Interaction of mammalian ABCD proteins with Arabidopsis Pex19 in vivo**
Tobacco plants stably expressing CFP-SKL were co-transfected with 35S::ABCD-YFP fusions and NLS-Pex19 constructs. Leaf epidermal cells were imaged using confocal microscopy: (A-D) ALDP-YFP plus NLS-HsPex19; (E-H) ALDP-YFP plus NLS-AtPex19_1; (I-J) ALDR-YFP plus NLS-AtPex19_1. (A,E,I) CFP; (B,F,J) YFP; (C,G,K) bright field; (D,H,L) merge. Scale bars: 10 μm. Results are representative of three independent experiments.

Figure 4. Low-temperature rescue of X-ALD fibroblasts
(A) ALDP expression levels in fibroblasts bearing X-ALD missense mutations, cultured at 37°C and at 30°C for 7 d. Asterisks indicate statistically significant differences, as determined by an unpaired t-test (* = P < 0.05, ** = P < 0.01, *** = P < 0.001). (B) Representative western blot, showing increase in ALDP in selected fibroblasts following culture at 30°C for 7 d. (C) VLCFA β-oxidation capacity in control fibroblasts from two different individuals and missense mutant ALDP-expressing fibroblasts cultured at 37°C or at 30°C for 3 d. Values are means ± SD; n=3. (D) Ratio of C26:0/C22:0 in control fibroblasts and missense mutant ALDP expressing fibroblasts cultured at 30°C for the time indicated. Values are means ± SD; n=3.

Figure 5. Targeting of missense X-ALD mutants in plant cells
Tobacco plants stably expressing CFP-SKL were transfected with 35S::ABCD-YFP fusions bearing X-ALD mutations reported in [58] and in this study (Fig. 4): p.Arg104Cys, p.Tyr174Cys, p.Ser606Leu, p.Ala616Thr, p.His667Asp. Leaf epidermal cells were imaged using confocal microscopy. (A) CFP (B) YFP (C) bright field (D) merge. Scale bars: 10 μm. Images are representative of results from three independent experiments.

Figure 6. Expression of ALDP-YFP and ALDR-YFP in cts-1
(A) Germination and subsequent seedling establishment of wild type (Ler), cts-1, and cts-1 transgenic lines expressing ALDP-YFP and ALDR-YFP. Images were recorded after 5 d on 0.5 X MS medium. Note the radicle emergence in cts-1/ALDR. (B) Seedling establishment analysed independently of germination: cts-1 and cts-1/ALDP seeds were induced to germinate by mechanical disruption of the seed coat and growth for one day on 0.5 X MS medium containing 1 % (w/v) sucrose, then transferred to 0.5 X MS medium for a further 4 d (C-E) Growth of seedlings on 0.5 X MS medium containing 1 % (w/v) sucrose. cts-1 and cts-1/ALDP-YFP seeds were induced to germinate by mechanical disruption of the seed coat. (C) no addition; (D) 1 μM 2,4-DB, (E) 30 μM IBA. Scale bar: 5 mm. Inset panels in (A) show seeds magnified 10 times. Results are representative of several independent transgenic lines.

Figure 7. Fatty acid profile of seedlings expressing mammalian ABCD transporters
Seedlings were cultured for 5 d on 0.5 X MS medium containing 1 % (w/v) sucrose. cts-1 and cts-1/ALDP-YFP seeds were induced to germinate by mechanical disruption of the seed coat. Fatty acid methyl esters were determined for whole seedlings. Bars represent means ± SD (n =3).
### Table 1. Quantification of ALDP levels in X-ALD fibroblasts

<table>
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<tr>
<th>Mutation</th>
<th>ALDP</th>
<th>Immunofluorescence</th>
<th>Immunoblot (% control)</th>
</tr>
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<tr>
<td>p.Arg74Trp</td>
<td>Absent</td>
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<td>7.5 ± 0.6</td>
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<tr>
<td>p.Arg104Cys</td>
<td>Reduced</td>
<td></td>
<td>35 ± 3.0</td>
</tr>
<tr>
<td>p.Ser149Asn</td>
<td>Present</td>
<td></td>
<td>77 ± 3.0</td>
</tr>
<tr>
<td>p.Asp194His</td>
<td>Present</td>
<td></td>
<td>60 ± 13.6</td>
</tr>
<tr>
<td>p.Leu220Pro</td>
<td>Reduced</td>
<td></td>
<td>21.8 ± 5.4</td>
</tr>
<tr>
<td>p.Arg389His</td>
<td>Present</td>
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<td>40.6 ± 3.6</td>
</tr>
<tr>
<td>p.Arg554His</td>
<td>Absent</td>
<td></td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>p.Ser606Leu</td>
<td>Present</td>
<td></td>
<td>25 ± 1.5</td>
</tr>
<tr>
<td>p.Glu609Gly</td>
<td>Absent</td>
<td></td>
<td>2.1 ± 1.3</td>
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<tr>
<td>p.Glu609Lys</td>
<td>Absent</td>
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<td>p.Ala616Thr</td>
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<td>4.3 ± 1.7</td>
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<td>p.Leu654Pro</td>
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<td>1.5 ± 1.3</td>
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<td>p.Arg660Trp</td>
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<td></td>
<td>1.6 ± 0.8</td>
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<td>p.His667Asp</td>
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<td>2.9 ± 1.0</td>
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<tr>
<td>p.Arg113fs</td>
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Table 2. X-ALD mutants used for analysis of targeting in tobacco cells

<table>
<thead>
<tr>
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<th>position</th>
<th>Protein fate in fibroblasts/CHO cells</th>
<th>Occurrencea</th>
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<tr>
<td>p.Arg104Cys</td>
<td>TMD1</td>
<td>Degraded in CHO cells, no MG-132 rescue1. Protein increased by low temperature.2</td>
<td>n=6, protein reduced/absent</td>
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<tr>
<td>p.Tyr174Cys</td>
<td>3 residues upstream of coupling helix 1</td>
<td>Mistargeted1</td>
<td>n=12, protein absent</td>
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<tr>
<td>p.Arg554His</td>
<td>NBD, 3 residues downstream of Q loop</td>
<td>Protein absent, rescued by low temperature.2</td>
<td>n=25, protein absent</td>
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<tr>
<td>p.Ser606Leu</td>
<td>NBD, ABC motif</td>
<td>Degraded, rescued by MG-1321; little effect of low temperature.2</td>
<td>n=12, protein present (p.Ser606Pro; n=3, absent)</td>
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<tr>
<td>p.Glu609Gly</td>
<td>NBD, ABC motif</td>
<td>Protein absent, increased by low temperature; no functional rescue.2</td>
<td>n=1 protein absent (p.Glu609Lys; n=14, absent)</td>
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<td>p.Ala616Thr</td>
<td>NBD, between ABC and Walker B</td>
<td>Protein absent, rescued by low temperature.2</td>
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<td>p.Arg660Trp</td>
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<td>p.His667Asp</td>
<td>NBD, 5 residues downstream of H-loop</td>
<td>Degraded, rescued by MG-1321; little effect of low temperature.2</td>
<td>n=3, protein absent</td>
</tr>
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a. Number of documented patients bearing the mutation; source: X-ALD database (www.x-ald.nl)

References: (1) [58]; (2) this study.
Abbreviations: TMD, transmembrane domain; NBD, nucleotide binding domain.
Table S1. Primers used for plasmid construction

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<td>ABCD2attB1 GGGAACGTTGACGTACAAAAAGCAGGCTAACATTGCGTCCCG</td>
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<td>ABCD2attB1 GGGAACGTTGACGTACAAAAAGCAGGCTAACATTGCGTCCCG</td>
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<td>ABCD3attB1 GGGAACGTTGACGTACAAAAAGCAGGCTAACATTGCGTCCCG</td>
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<td><strong>NLS construct primers</strong></td>
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<td>H2BAtPex19attB2 GGGAACGTTGACGTACAAAAAGCAGGCTAACATTGCGTCCCG</td>
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<td>FT123 GAGGATCCATGGCGAACAGTCACACCCGAT</td>
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<td>FT125 CAGGATCCACATGACATGATCGAC</td>
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<td>FT126 CAGGATCCACATGACATGATCGAC</td>
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<td><strong>Primers for site-directed mutagenesis</strong></td>
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**Primers for quantitative RT-PCR of ABCD1 mRNA**

**Primers for quantitative RT-PCR of ABI5 mRNA**
Fig. 2

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</table>

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Fig. 4

A

ALDP (% of control fibroblast)

30°C

37°C

Arg74Trp

Arg104Cys

Ser149Asn

Asp194His

Leu220Pro

Arg389His

Arg554His

Ser606Leu

Glu609Gly

Glu609Lys

Ala616Thr

Leu654Pro

Arg660Trp

His667Asp

Arg113fs

B

C

VLCFA beta-oxidation activity (100% = 37°C control)

Control1

Control107

Arg660Trp

Ala616Thr

Glu609Gly

Arg554His

Asp194His

Arg113fs

D

C26:0 / C22:0

Time (weeks)

0

1

2

3

4

Glu609Gly

Arg113fs

Asp194His

Arg660Trp

Arg554His

Ala616Thr

Control1

Control107
Fig. 5

Arg104Cys

Tyr174Cys

Ser606Leu

Ala616Thr

His667Asp
Fig. 6

A  
Ler  cts-1  cts-1: ALDP  cts-1: ALDR

B

C

D

E