

# Substrate Specificity Overlap and Interaction between Adrenoleukodystrophy Protein (ALDP/ABCD1) and Adrenoleukodystrophy-related Protein (ALDRP/ABCD2)\*

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X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disorder caused by mutations in the *ABCD1* gene, which encodes a peroxisomal member of the ATP-binding cassette (ABC) transporter subfamily D called ALDP. ALDP is supposed to function as a homodimer allowing the entry of CoA-esters of very-long chain fatty acids (VLCFA) into the peroxisome, the unique site of their  $\beta$ -oxidation. ALDP deficiency can be corrected by overexpression of ALDRP, its closest homolog. However, the exact nature of the substrates transported by ALDRP and its relationships with ALDP still remain unclear. To gain insight into the function of ALDRP, we used cell models allowing the induction in a dose-dependent manner of a wild type or a mutated non-functional ALDRP-EGFP fusion protein. We explored the consequences of the changes of ALDRP expression levels on the fatty acid content (saturated, monounsaturated, and polyunsaturated fatty acids) in phospholipids as well as on the levels of  $\beta$ -oxidation of 3 suspected substrates: C26:0, C24:0, and C22:6n-3 (DHA). We found an inverse correlation between the fatty acid content of saturated (C26:0, C24:0) and monounsaturated (C26:1, C24:1) VLCFA and the expression level of ALDRP. Interestingly, we obtained a transdominant-negative effect of the inactive ALDRP-EGFP on ALDP function. This effect is due to a physical interaction between ALDRP and ALDP that we evidenced by proximity ligation assays and coimmunoprecipitation. Finally, the  $\beta$ -oxidation assays demonstrate a role of ALDRP in the metabolism of saturated VLCFA (redundant with that of ALDP) but also a specific involvement of ALDRP in the metabolism of DHA.

X-linked adrenoleukodystrophy (X-ALD)<sup>2</sup> (OMIM 300100) is a very complex neurodegenerative disorder whose pathogenesis remains to be clarified (1). X-ALD is caused by

mutations in the *ABCD1* gene located in Xq28, which encodes a peroxisomal member of the ATP-binding cassette (ABC) transporter subfamily D called ALDP (adrenoleukodystrophy-protein) (2). This protein has the structure of a half ABC transporter, which is supposed to function as a homodimer (3, 4). However, heterodimerization with one of the other members of the ABCD subfamily (*i.e.* ALDRP (encoded by the *ABCD2* gene (5)), PMP70 (*ABCD3* (6)), and PMP69 (*ABCD4* (7, 8))) cannot be excluded especially in the situation where these proteins are overexpressed. Although a mirror expression pattern is often observed between ALDP and ALDRP when specific cell types are analyzed (9), peroxisomal ABC transporters have overlapping expression patterns rendering possible such interactions (5, 10). Coimmunoprecipitation experiments or FRET analysis have demonstrated heterodimerization in cells overexpressing the peroxisomal ABC transporters (11, 12). Although the peroxisomal localization of ALDP, ALDRP, and PMP70 is clearly demonstrated, PMP69 has recently been described to be localized in the endoplasmic reticulum and was found to be absent in the peroxisome. This excludes a possible interaction at the peroxisomal membrane with the other ABC transporters (13). Based on the model of the transport of pigment precursors in *Drosophila* (14), differences in the relative expression level of each peroxisomal ABC transporter in a single cell type could lead to alternative dimerization and consequently to a change in substrate specificity.

Defective peroxisomal  $\beta$ -oxidation and accumulation of saturated and monounsaturated very-long-chain fatty acids (VLCFA) are the main biochemical features of X-ALD. This observation as well as recent work in yeast (3) let suppose that ALDP participates in the entry of CoA-esters of VLCFA into the peroxisome, the unique site of their  $\beta$ -oxidation. In fibroblasts, the  $\beta$ -oxidation defect due to ALDP deficiency is partially corrected by overexpression of PMP70 and fully restored by overexpression of ALDRP (15, 16). Moreover, this partial functional gene redundancy is also recognized *in vivo* because reversion of the adrenomyeloneuropathy-like phenotype has been observed in *Abcd1* null mice overexpressing *Abcd2* in an ubiquitous manner (17). Overexpression of ALDRP has been demonstrated to prevent VLCFA accumulation and the onset of a neurological phenotype. Therefore, both *ABCD2* and *ABCD3* genes constitute potential therapeutic targets for X-ALD in a strategy aimed at inducing their expression through

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<sup>2</sup> The abbreviations used are: X-ALD, X-linked adrenoleukodystrophy; ABC, ATP-binding cassette; ALDP, adrenoleukodystrophy protein; ALDRP, adrenoleukodystrophy-related protein; DHA, docosahexaenoic acid; IF, immunofluorescence; PLA, proximity ligation assay; PMP, peroxisomal membrane protein; PUFA, polyunsaturated fatty acids; VLCFA, very-long-chain fatty acids; PC, phosphatidylcholine.

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pharmacological treatments. Concerning the substrates, an overlap in the substrate specificity of ALDP, ALDRP, and PMP70 is likely. Nevertheless, lessons from the different knockout mice models suggest that PMP70 would preferentially be dedicated to the transport of branched-chain fatty acids and bile acid precursors (18), whereas ALDRP would play a role in the catabolism of long-chain saturated and monounsaturated fatty acids and in the synthesis of DHA (C22:6*n*-3) (19). However, the exact nature of the substrates transported by ALDRP and PMP70, their relationship with ALDP, and their state of dimerization (homo- or heterodimerization) absolutely needs further investigations particularly in situations of induced over-expression, a situation found in pharmacological induction based therapies.

In this study, we focused on the role of ALDRP and its capability to influence the function of ALDP. We used previously described cell models that allow to induce the expression of a wild type or a mutated non-functional ALDRP-EGFP (enhanced green fluorescent protein) fusion protein in a dose-dependent manner (20). These cells are derived from H4IIEC3 hepatoma cells in which ALDRP is almost not expressed in normal conditions, whereas ALDP and PMP70 are highly expressed. We explored the consequences of changes of the ALDRP expression level on fatty acid content in phospholipids (PL) as well as on the levels of  $\beta$ -oxidation of 3 suspected substrates: C26:0, C24:0, and C22:6*n*-3. We also explored the dimeric status of ALDRP by coimmunoprecipitation and proximity ligation assay.

### EXPERIMENTAL PROCEDURES

**Cell Culture**—H4IIEC3 cells (rat hepatoma cells, ATCC: CRL-1600) were cultured in DMEM/Ham's F-12 (1/1) supplemented with 5% FCS at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> in the absence of antibiotics. WT (clone 28) and D207H (clone 19) ALDRP-EGFP Tet-on cell clones were established as described previously (20), and cultured in the presence of 200  $\mu$ g/ml of G418 (InvivoGen) and 200  $\mu$ g/ml of hygromycin B (InvivoGen). Initial cell clones 19 and 28 have been subcloned by limiting dilution in poly-L-lysine-coated 96-well plates in tissue culture medium conditioned by H4IIEC3 cells. Cell clones 19 and 28 were seeded (0.05, 0.5, or 5 cells/well) in 96-well plates in conditioned culture medium supplemented with 200  $\mu$ g/ml of G418 and 200  $\mu$ g/ml of hygromycin B. The medium was changed weekly. Approximately 3 weeks later, cells from single-colony containing wells were transferred to 24-well plates prior to subsequent analysis. To further select subclones expressing the highest expression levels of WT or D207H Abcd2-EGFP, cells were incubated or not for 24 h with 2  $\mu$ g/ml of doxycycline (Clontech). Then, cells were fixed in 1% paraformaldehyde and the level of doxycycline-induced EGFP fluorescence was analyzed by fluorescence-activated cell sorting (FACS) and Western blotting. Finally, subclones 28.38 and 19.55 were selected.

**Cell Treatment with Solubilized VLCFA**—The desired amounts of C26:0 (Larodan Fine Chemicals AB) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC) (Sigma) dissolved in CHCl<sub>3</sub> were incubated together (1/4, w/w). The solvent was further evaporated under a stream of dry nitro-

gen. The C26:0/PC mixture was hydrated with H<sub>2</sub>O and sonicated for 30 min at 4 °C with a sonifier tip (Vibra Cell, Bio-block). The PC vesicles containing C26:0 fatty acid were centrifuged for 5 min at 20,000  $\times$  *g* and then gently mixed at 37 °C with aqueous bovine serum albumin (BSA, dissolved in 0.9% NaCl) to achieve 2 mol of fatty acid/mol of BSA.  $4.3 \times 10^6$  cells were seeded in 21.5-cm<sup>2</sup> culture flasks and cultured in DMEM/Ham's F-12 (1/1) supplemented with 5% FCS, 200  $\mu$ g/ml of G418 and hygromycin B for 48 h. Then, cells were washed twice in PBS and deprived of FCS, preincubated for 3 h in the presence or absence of various doses of doxycycline (0.1, 0.5, or 1  $\mu$ g/ml) and cultured in the same conditions for the following 15 h in the presence or absence of 25  $\mu$ M C26:0 (C26:0/PC mixture). Each point was performed in quadruplet to allow RT-qPCR, protein (Western blotting and fluorescence microscopy), and lipid analysis.

**GC-MS Analysis**—After treatment of cell clones with or without doxycycline and or a specific fatty acid, cell nuclei were removed by centrifugation at 1,000  $\times$  *g* for 10 min at 4 °C. Cellular lipids were extracted with chloroform/methanol (2/1, v/v) according to the method of Folch (35). Dihenarachidoyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) was added as an internal standard. Phospholipids were separated from other lipid classes by HPLC (HP1100, Agilent Technologies) on a 200  $\times$  2.1-mm, 5- $\mu$ m Hypersil Silica column (Thermo) with a binary gradient of chloroform/methanol (8/2, v/v) and chloroform/methanol/H<sub>2</sub>O (6/3.4/0.6, v/v/v). Phospholipids containing fractions were collected, evaporated under nitrogen, and hydrolyzed with methanolic potassium hydroxide. Fatty acids were analyzed as pentafluorobenzyl esters by gas chromatography/negative chemical ionization mass spectrometry as previously described (30) using a HP7890A gas chromatograph equipped with an HP7683 injector and a HP5975C mass selective detector (Agilent Technologies). Chromatography was performed using an HP5MS fused silica capillary column (30 m  $\times$  0.25-mm inner diameter, 0.25- $\mu$ m film thickness, Agilent Technologies).

**Fatty Acid  $\beta$ -Oxidation Assays**—H4IIEC3 cell clones were seeded in sterile glass vials at  $4 \times 10^5$  cells in 3 ml of culture medium supplemented with 200  $\mu$ g/ml of G418, 200  $\mu$ g/ml of hygromycin B, and 2  $\mu$ g/ml of doxycycline or not. After 48 h at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>, the culture medium was removed, cell clones were washed twice with PBS, and  $\beta$ -oxidation analysis were performed, as described previously (21). C26:0, C24:0 and C22:6*n*-3 were purchased from Larodan Fine Chemicals AB and dissolved in CHCl<sub>3</sub>. The solvent was evaporated under a stream of dry nitrogen. Then, VLCFA were incubated with  $\alpha$ -cyclodextrin (Sigma) to achieve 85 mol of  $\alpha$ -cyclodextrin/mol of C26:0, C24:0, or C22:6*n*-3. The solubilized VLCFA were sonicated with a sonifier bath for 1 h. The assay was initiated by the addition on the cells of 4  $\mu$ M [1-<sup>14</sup>C]docosahexaenoic acid (C22:6*n*-3), [1<sup>14</sup>C]tetracosanoic acid (C24:0), or [1-<sup>14</sup>C]hexacosanoic acid (C26:0) with 8  $\mu$ M of the corresponding non-radiolabeled fatty acid. Radiolabeled fatty acids were purchased from American Radiolabeled Chemicals. The sum of radioactive [<sup>14</sup>C]CO<sub>2</sub> and <sup>14</sup>C-labeled acid-soluble products was taken as a measure of fatty acid oxidation and rates were expressed as picomole/h per mg of pro-

tein. Total proteins were quantified according to the Bradford protein assay (usually between 200 and 300 mg/vial).

**Immunofluorescence (IF) and in Situ Proximity Ligation Assay (PLA)**—H4IIEC3 cell clones were seeded at  $2 \times 10^5$  per well in 16-well Lab-Tek chambers slides (Nunc) pre-coated with 0.1 mg/ml of poly-L-lysine. After an attachment period of 24 h, cells were treated or not with doxycycline (2  $\mu$ g/ml) and incubated during 48 h at 37 °C. Then, they were washed with PBS, fixed in 4% paraformaldehyde, pH 7.5, for 10 min at RT, rinsed in PBS, and incubated two times in PBS, 0.1 M glycine for 5 min. Cells were permeabilized for 10 min at RT with FACS permeabilizing solution (BD Biosciences), rinsed three times in PBS, 0.05% saponin and incubated during 20 min at RT with PBS, 0.05% saponin, 5% rat serum to saturate Fc receptors.

The following antibodies were used in IF detection or in PLA assay: 1/500 diluted mouse anti-GFP antibody (Roche Applied Science), 1/1,000 diluted rabbit polyclonal anti-ALDP antibody (22), 1/200 diluted rabbit anti-PEX14 (gift from M. Fransen), and 1/100 diluted rabbit polyclonal anti-PMP22. In IF assays, cells were incubated in the presence of primary antibodies in PBS, 0.05% saponin for 20 min, washed 3 times in PBS, 0.05% saponin, incubated 20 min with the 1/1,000 diluted Alexa 594 goat anti-mouse or anti-rabbit secondary antibody (Invitrogen) and finally washed 3 times in PBS before mounting in fluorsave (Merck). In PLA assays, cells were incubated in the presence of a convenient couple of primary antibodies diluted in PBS, 0.05% saponin, 2% rat serum for 20 min at RT. Incubation with appropriate PLA probe (anti-mouse or anti-rabbit antibody), hybridization, amplification, development, and slide preparation were done using the DuoLink kit (Olink Biosciences) following the manufacturer's instructions. Negative control experiments (either one or both primary antibodies were omitted) were performed in parallel and checked to result in the absence of PLA signal.

Finally, the samples were mounted with DuoLink Mounting Medium. IF and PLA slides were analyzed in confocal microscopy using TCS SP2 AOBS confocal laser microscope (Leica Microsystems SA) and a  $\times 40$  oil immersion objective. Image processing and analysis were done using the Leica confocal software.

**Coimmunoprecipitation Assays and Western Blotting**—H4IIEC3 cells stably expressing D207H-ALDRP-EGFP after overnight induction with 2  $\mu$ g/ml of doxycycline or not were homogenized in solubilization buffer (100 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% PMSF and protease inhibitor mixtures (Roche Applied Science)). Nuclei were removed by centrifugation for 10 min at 4 °C and  $1,000 \times g$ . The cell lysate was incubated twice with agarose beads for 1 h at 4 °C with gentle rotation. After this pre-clearing step, lysate was incubated with rabbit polyclonal anti-ALDP antibody (22) cross-linked to agarose beads via dimethyl pimelimidate (protocol adapted from Ref. 23) or mouse monoclonal anti-GFP antibody covalently immobilized on agarose beads (MBL) for 1 h at 4 °C with gentle rotation. The beads were washed three times with wash buffer (100 mM Tris-HCl, pH 8, 100 mM NaCl, 10 mM EDTA, and 1% Triton X-100), and bound proteins were released with Laemmli buffer. Immunoprecipitated proteins or proteins from total cell lysate were separated on a 7.5% SDS-PAGE and transferred onto a PVDF membrane. The membrane

was first incubated in 5% skimmed milk dissolved in PBS, 0.1% Tween 20 (PBS/T) for 1 h at room temperature. The membrane was probed with 1/500 diluted mouse anti-GFP antibody (Roche Applied Science) or with rabbit polyclonal anti-ALDP (1/5,000) antibody (22) and then with the appropriate secondary antibody coupled to horseradish peroxidase (1/5,000 Santa Cruz Biotechnology), before being revealed by ECL (Santa Cruz Biotechnology).

**RT-qPCR**—Total RNA was extracted using RNeasy Mini kit (Qiagen) following the manufacturer's instructions. cDNA was generated using Moloney murine leukemia virus reverse transcriptase enzyme (Promega). Quantification of cDNA expression was carried out using the SYBR Green real time PCR technology and an iCycler iQ Real Time Detection System (Bio-Rad) as previously described (24). The primers 5'-GGCATCACCACTAAAATCTCC-3' (rat *36b4* forward), 5'-GCTCCCACCTTGCTCTCCA-3' (rat *36b4* reverse), 5'-CAGCGTCCACTTACCACATAG-3' (rat *Abcd2* forward), and 5'-CGTCAGCAATGCGTACTTTCG-3' (rat *Abcd2* reverse) were chosen using the Beacon Designer Software (Bio-Rad). Quantitative analysis of *Abcd2* expression was performed using cycle to threshold ( $C_t$ ) values and normalized by the *36b4* reference gene encoding for the acidic ribosomal phosphoprotein P0.

**Statistical Analyses**—Statistical analyses were performed by Mann-Whitney test using the StatView statistical software. The differences between the treated and the control groups are indicated as significant ( $p < 0.05$ ) or highly significant ( $p < 0.01$ ).

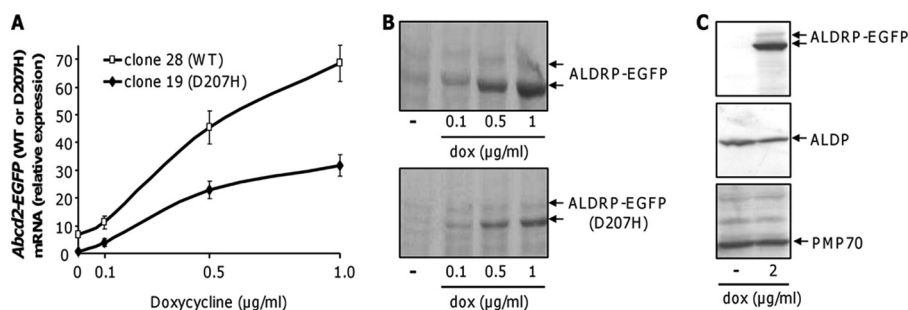
## RESULTS

**Induction Level of ALDRP-EGFP Fusion Protein in the Cell Clones**—To gain insight into the function of ALDRP, we used specific cell models specifically engineered to express a mutant (clone 19) or a wild type (clone 28) ALDRP-EGFP fusion protein depending on the dose of doxycycline added in the culture medium. The WT ALDRP-EGFP fusion protein was proven to be functional, at least concerning the capability to restore the  $\beta$ -oxidation of C26:0 in X-ALD fibroblasts (20). The mutant ALDRP-EGFP corresponds to a single change of the amino acid sequence (D207H) mimicking a natural mutation occurring in the *ABCD1* gene (D196H ALDP), which results in a non-functional protein still able to dimerize and to be targeted at the peroxisomal membrane (25).

As seen at the mRNA level quantified by RT-qPCR (Fig. 1A) and at the protein level observed by Western blotting (Fig. 1B), both cell clones respond to doxycycline treatment (18 h) in a dose-dependent manner. It is noteworthy that treatment with doxycycline results in the induction of *Abcd2-EGFP* and does not modify ALDP or PMP70 expression in clones 28 (Fig. 1C) and 19 (data not shown). Although the basal level of the fusion protein is quasi null in clone 19 (not different from H4IIEC3 control cells), a leaky expression of ALDRP-EGFP was observed in clone 28. Therefore, the relative induction appears to be stronger in clone 19 than in clone 28.

**Inverse Correlation between the Level of Expression of the WT ALDRP-EGFP Fusion Protein and Saturated VLCFA Content in Phospholipids**—Changes in lipid composition and fatty acid metabolism have been largely described in

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**FIGURE 1. Doxycycline-dependent expression of the WT or D207H *Abcd2-EGFP* gene in stable transfectant cell clones 28 and 19, respectively.** Cell clones were cultivated 18 h in the presence of various doses of doxycycline. Expression was quantified at the mRNA level by RT-qPCR (A) and at the protein level by Western blotting analysis with anti-GFP antibody (B). ALDRP-EGFP expression does not modify ALDP nor PMP70 expression (C). Clone 28 cell lysates were separated on 7.5% SDS gels, blotted, and probed with anti-GFP, anti-ALDP, or anti-PMP70 antibodies.

several models of peroxisomal alterations, in particular in ALDP-deficient cells. These modifications are perfectly visible in PL.

Peroxisomes, but especially mitochondria, participate in degradation of fatty acids. Therefore, the consequences of the alterations of peroxisomal import, if any, were expected to be underestimated for most of the fatty acids with the exception of VLCFA, which are  $\beta$ -oxidized only into the peroxisome. Moreover, because the alteration of ALDRP (WT or D207H) expression was unlikely to result in strong modifications in normal conditions (ALDP and PMP70 are quite well expressed in H4IEC3 cells), we challenged the cells with C26:0, the supposed preferential substrate of ALDP, and compared the different situations. Fatty acid supplementation of hepatoma cells have been demonstrated to result in rapid modification of the membrane content (26). The kinetic of such a supplementation being compatible with the kinetic of doxycycline-dependent induction, we decided to analyze the fatty acid content in PL after an 18-h incubation with doxycycline and in the presence or not of C26:0 for 15 h. It is important to notice that the ALDRP-EGFP expression level was not modified by fatty acid supplementation.

GC-MS analysis of the fatty acid content in PL was performed in triplicate from 5 independent experiments. We first quantified saturated fatty acids (C18:0, C20:0, C22:0, C24:0, and C26:0). We observed a dose-dependent decrease of the relative level of C26:0 (reaching a maximum of 0.79-fold) and C24:0 (maximum of 0.78-fold) in clone 28 (Fig. 2). A clear inverse correlation was thus observed between the level of expression of the WT ALDRP-EGFP protein and the saturated VLCFA content in PL. No significant variation was seen for shorter fatty acids, C18:0-C22:0 (Fig. 2). Incubation of the cells with C26:0 resulted in a 1.66-fold increase of the C26:0 content in PL but no significant changes for the other fatty acids. In the presence of C26:0, a significant doxycycline-dependent decrease was observed only for the C26:0 and C24:0 levels (Fig. 2).

**Transdominant Negative Effect of D207H-ALDRP-EGFP—**Parallel experiments were conducted with cell clone 19 expressing the mutant non-functional D207H-ALDRP-EGFP protein. As shown in Fig. 2, no significant variation was observed for C24:0, C22:0, C20:0, and C18:0. However, whereas induction of ALDRP-EGFP in clone 28 resulted in a decreased content of C26:0 and C24:0, doxycycline-dependent induction of D207H-ALDRP-EGFP resulted in a dose-dependent increase

of the level of C26:0 (Fig. 2). Because the basal level of expression of the endogenous ALDRP in cell clones is quasi null, this result may be the consequence of a transdominant-negative effect of D207H-ALDRP-EGFP on ALDP function.

**D207H-ALDRP-EGFP Interacts with ALDP—**To evidence the interaction between D207H-ALDRP-EGFP and ALDP, we used two complementary approaches. First, we used a proximity ligation assay (PLA duolink) to analyze *in situ* the proximity of D207H-ALDRP-EGFP with ALDP. The technique is based on the use of primary antibodies obtained in different species. Secondary antibodies targeting either rabbit or mouse antibodies are coupled to oligonucleotides, which serve as proximity probes. If the distance between the proteins is less than 40 nm, probes can hybridize with subsequently added connector oligonucleotides and guide the formation of a circular DNA template, which is amplified and detected by fluorescence hybridization. This technique was successfully used to detect the dimerization of receptor tyrosine kinase (27). As a first step, we validated the primary antibodies used in the PLA experiment. For each antibody, a peroxisomal punctuate labeling colocalizing with the EGFP signal was obtained from immunofluorescence experiments performed on clone 19 cultivated in the presence of doxycycline (Fig. 3A). PLA experiments resulted in a positive labeling only in the presence of doxycycline and with the couple of antibodies directed against ALDP and GFP (Fig. 3B) demonstrating the proximity between ALDP and D207H-ALDRP-EGFP. This labeling does not result from the overabundance of proteins in a membrane context because no PLA labeling was obtained between D207H-ALDRP-EGFP and proteins PEX14 or PMP22, PMP22 being the most represented protein of the peroxisomal membrane (28).

To confirm that proximity between D207H-ALDRP-EGFP and ALDP is due to a physical interaction, cross-coimmunoprecipitation experiments were carried out from cell lysates obtained from clone 19 cultivated in the presence of doxycycline. As shown in Fig. 4A, D207H-ALDRP-EGFP was coimmunoprecipitated with ALDP by anti-ALDP antibody only in the doxycycline-treated cell clone 19. In the cross-experiment, ALDP was coimmunoprecipitated with D207H-ALDRP-EGFP by anti-GFP antibody only in doxycycline-treated cell clone 19 (Fig. 4B) demonstrating interaction. Similar results were obtained with the WT ALDRP-EGFP fusion protein and ALDP,

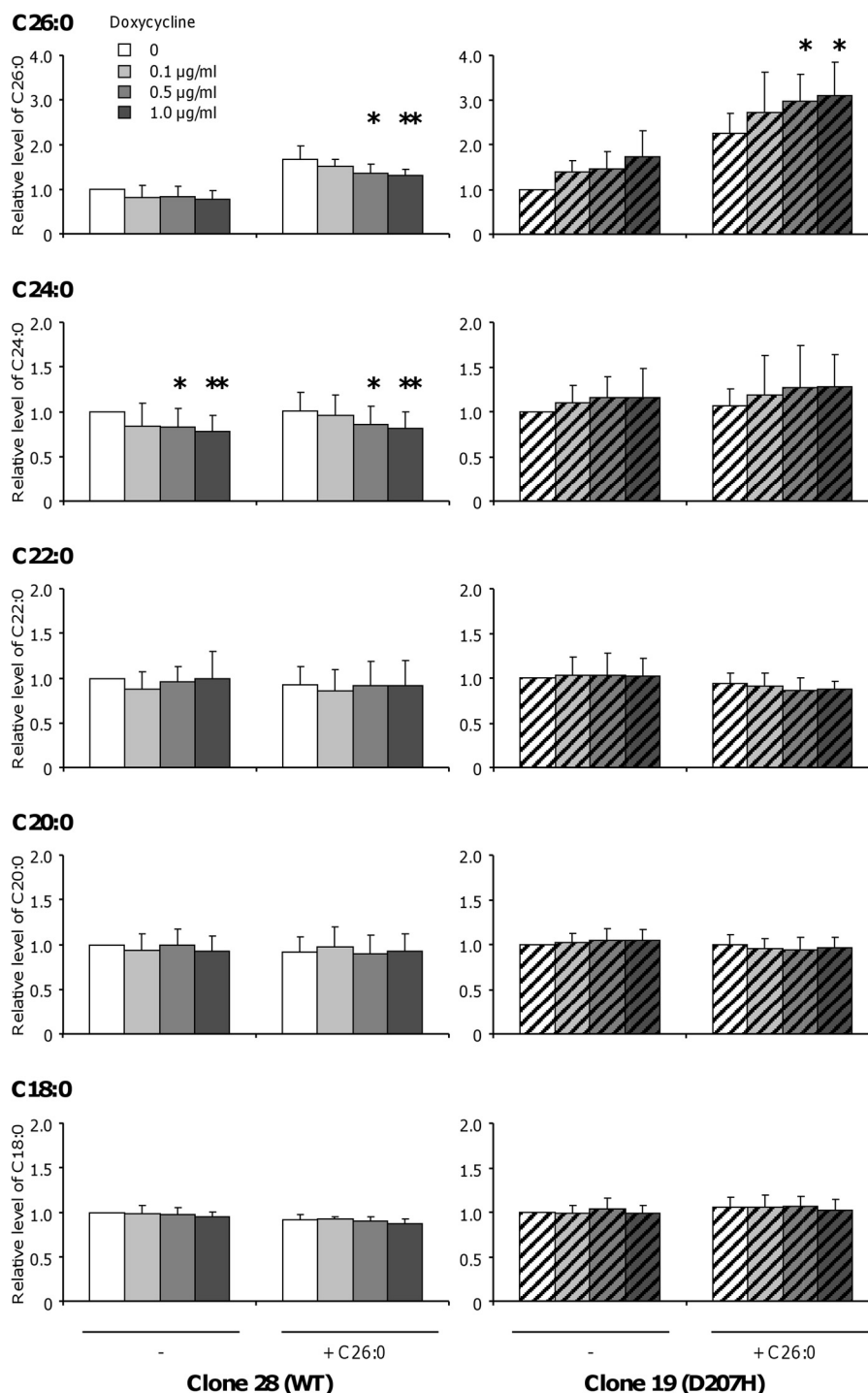


FIGURE 2. GC-MS analysis of the saturated fatty acid content in phospholipids of cell clones 28 (WT ALDRP-EGFP) and 19 (D207H-ALDRP-EGFP) cultivated in the presence of various doses of doxycycline and in the absence or presence of C26:0. Results of 5 independent experiments performed in triplicate are presented normalized to the percentage of fatty acid in the absence of doxycycline and C26:0 arbitrary taken equal to 1. Statistically significant variations are indicated by asterisks (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

indicating that interaction can occur independently of the mutation.<sup>3</sup>

*Inverse Correlation between the Level of Expression of the ALDRP-EGFP Fusion Protein and *n*-9 VLCFA Content in Phospholipids*—By GC-MS analysis, we further explored the fatty acid content in PL for *n*-7 and *n*-9 monounsaturated fatty

acids (18:1, 20:1, 22:1, 24:1, and 26:1) and polyunsaturated fatty acids (PUFA: C18:3, C20:3, C20:4, C20:5, C22:5, C22:6, and C24:6*n*-3; and C18:2, C18:3, C20:3, C20:4, C22:4, and C22:5*n*-6). Although we did not notice any change in the PUFA content nor in the *n*-7 monounsaturated fatty acid content whatever the conditions and the cell clones, the *n*-9 monounsaturated fatty acid content demonstrated alteration depending on the expression level of WT or D207H-ALDRP-EGFP protein (Fig. 5).

<sup>3</sup> F. Geillon, D. Trompier, C. Gondcaille, and S. Savary, manuscript in preparation.

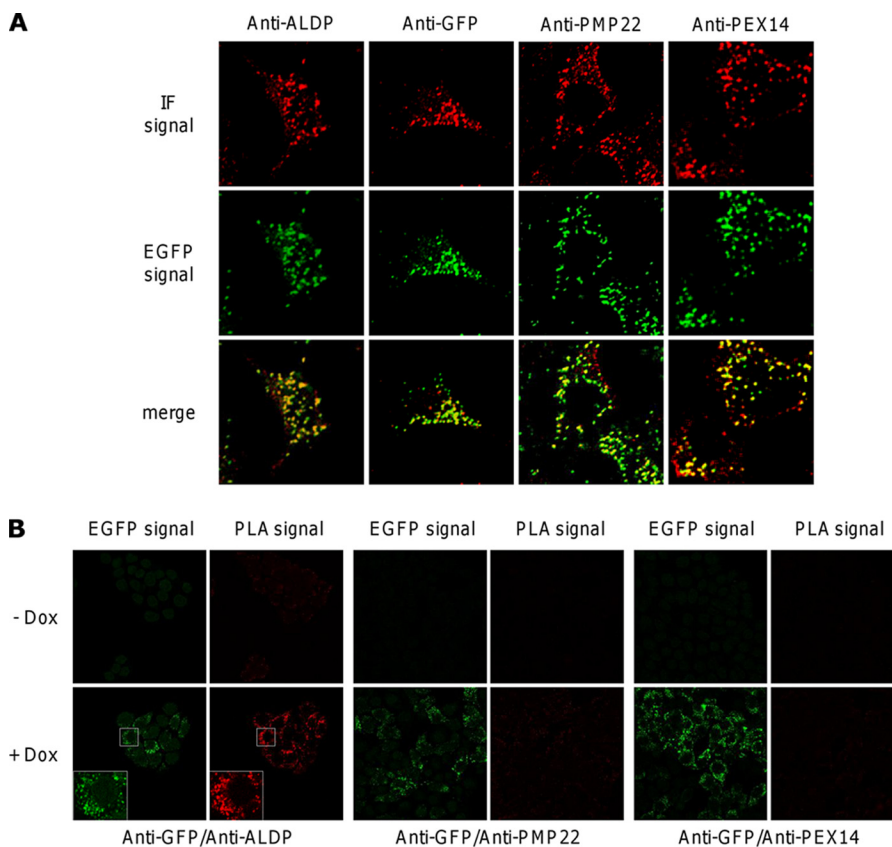


FIGURE 3. *In situ* analysis of the proximity of ALDRP-EGFP with other peroxisomal proteins. *A*, immunofluorescence detection (IF signal) of D207H-ALDRP-EGFP, ALDP, PEX14, and PMP22 observed by confocal microscopy in clone 19 (D207H-ALDRP-EGFP) induced by doxycycline (DOX) (2  $\mu$ g/ml for 48 h). *B*, Duolink proximity ligation assay between D207H-ALDRP-EGFP and ALDP, PEX14, and PMP22 in clone 19 induced by doxycycline observed by confocal microscopy.

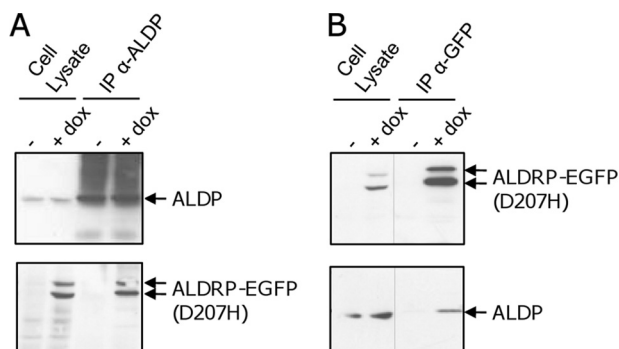


FIGURE 4. **Coimmunoprecipitation of ALDP and D207H-ALDRP-EGFP demonstrating interaction.** Cell lysates from cell clone 19 (D207H-ALDRP-EGFP) treated or not with doxycycline (*dox*) (2  $\mu$ g/ml) for 18 h were used for anti-ALDP (*A*) or anti-GFP (*B*) immunoprecipitation. Aliquots of cell lysates and eluted fractions were separated on 7.5% SDS gels, blotted, and probed with anti-ALDP or anti-GFP antibodies.

Incubation in the presence or absence of C26:0 yielded quite similar results (Fig. 5). C26:1*n*-9 levels followed the same pattern as saturated VLCFA. In the WT cell clone, we observed a significant doxycycline-dependent decrease of the C26:1*n*-9 content either in the absence or presence of C26:0 in the culture medium (Fig. 5). On the contrary, doxycycline-dependent induction of D207H-ALDRP-EGFP resulted in a dose-dependent increase of the level of C26:1*n*-9, although non-statistically significant. Thus, a transdominant-negative effect seems to be observed for C26:1. Interestingly, induction of ALDRP-EGFP resulted in a decreased content of C24:1*n*-9 but we did not

observe a transdominant-negative effect for this fatty acid in clone 19 (Fig. 5). No significant variation was observed for the shorter fatty acids analyzed (C18:1, C20:1, and C22:1*n*-9) (Fig. 5).

*Induction of ALDRP-EGFP Expression Leads to Increased  $\beta$ -Oxidation Levels of C26:0, C24:0, and C22:6*n*-3*—As induction of ALDRP-EGFP expression influence the cellular VLCFA content, we analyzed the  $\beta$ -oxidation level of commercially available  $^{14}$ C-labeled fatty acids: C26:0, C24:0, and C22:6*n*-3. Due to a relatively long delay between the first GC-MS analyses and these experiments of  $\beta$ -oxidation measurement, experiments were carried out in subclones of clones 19 and 28, respectively, clones 19.55 and 28.38. Indeed, the original clones demonstrated a progressive tendency to gene inactivation and subcloning by limited dilution was necessary. The obtained subclones displayed similar levels of induction as checked by flow cytometry and Western blotting. The  $\beta$ -oxidation levels in both cell clones were compared in the absence or presence of 2  $\mu$ g/ml of doxycycline. As shown in Fig. 6, doxycycline treatment of clone 28.38 resulted in a significant increase of the  $\beta$ -oxidation level of the 3 tested fatty acids. Concerning saturated VLCFA, this result was in agreement with results as mentioned before (Fig. 2) suggesting that induction of ALDRP-EGFP improves the peroxisomal entry of these fatty acids into the peroxisome, and therefore, leads to a decreased level of their contents in PL. However, the increased level of  $\beta$ -oxidation of DHA was unexpected because the levels of PUFA in PL appeared to be insensitive to the level of expression of ALDRP-

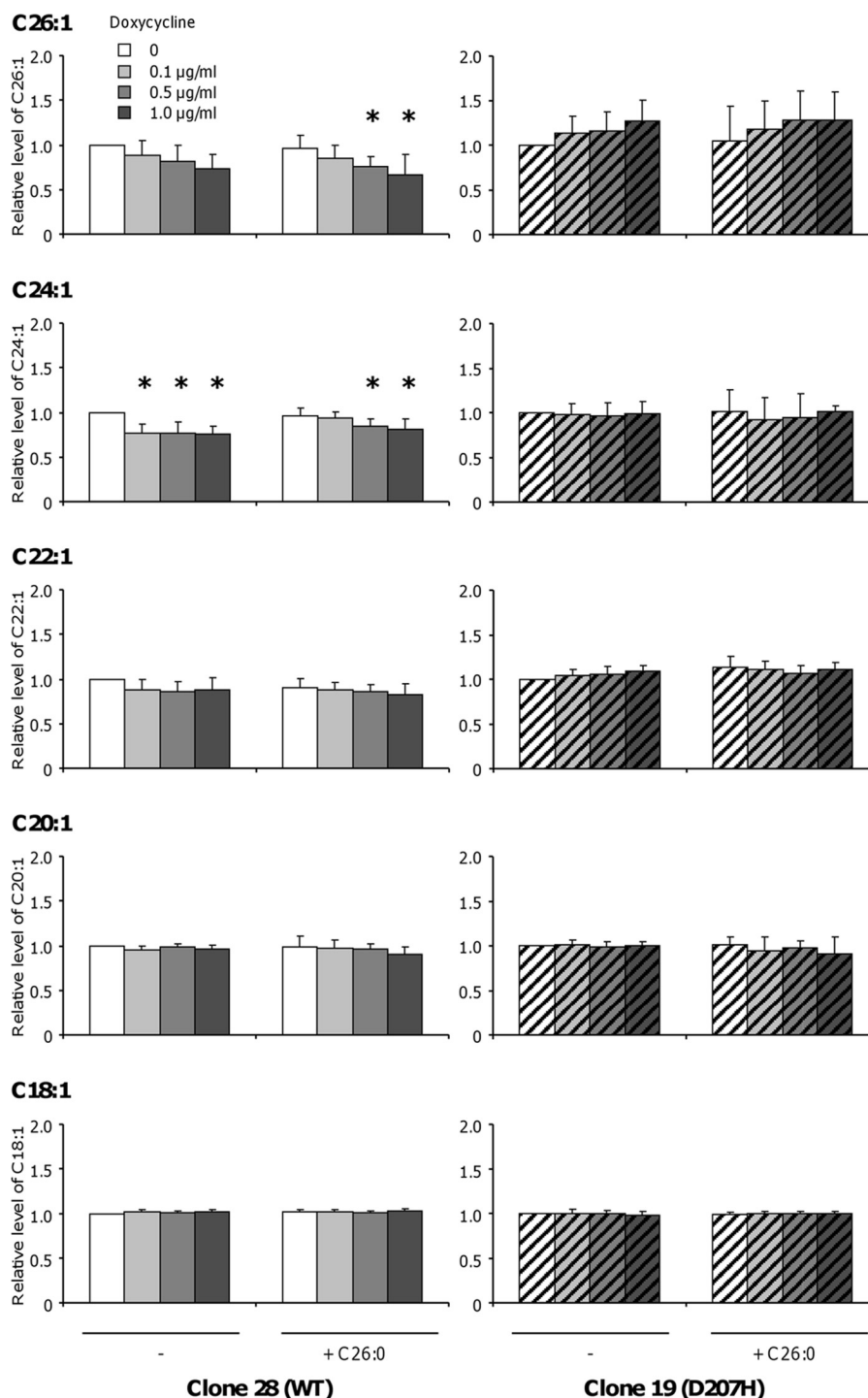


FIGURE 5. GC-MS analysis of the *n*-9 monounsaturated fatty acid content in phospholipids of cell clones 28 (WT ALDRP-EGFP) and 19 (D207H-ALDRP-EGFP) cultivated in the presence of various doses of doxycycline and in the absence or presence of C26:0. Results of 5 independent experiments performed in triplicate are presented normalized to the percentage of fatty acid in the absence of doxycycline and C26:0 arbitrary taken equal to 1. Statistically significant variations are indicated by asterisks (\*,  $p < 0.05$ ).

EGFP. Induction of D207H-ALDRP-EGFP resulted in a weak but significant decrease of the  $\beta$ -oxidation level of C26:0. However, we did not observe any influence on the C24:0 and C22:0  $\beta$ -oxidation levels.

## DISCUSSION

Pharmacological induction of *ABCD2* could represent an alternative therapeutic strategy for X-ALD patients. To develop

such a therapeutic approach, it is crucial to understand the exact function of ALDRP in normal or overexpressed conditions and compare its function to that of ALDP.

Although a defective transport is thought to trigger fatty acid accumulation, expression of ALDP or ALDRP in X-ALD fibroblasts restores the transport and then the degradation of the fatty acids accumulated (15, 16). The modified H4IIIEC3 cell clones cultivated in the absence or presence of increasing doses

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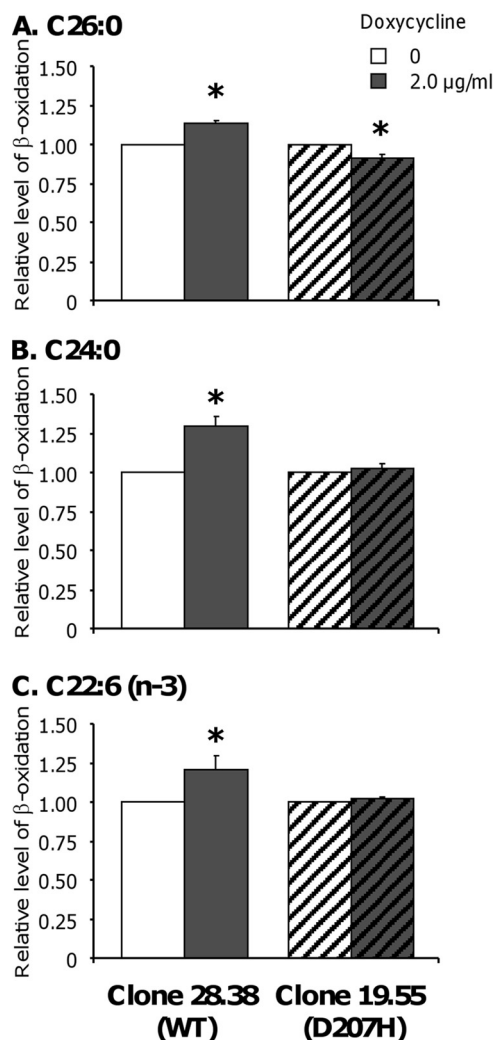


FIGURE 6.  $\beta$ -Oxidation levels of  $^{14}\text{C}$ -C26:0 (A), C24:0 (B), and C22:6n-3 (C) in clones 28.38 (WT ALDRP-EGFP) and 19.55 (D207H-ALDRP-EGFP) cultivated in the absence or presence of doxycycline (2  $\mu\text{g}/\text{ml}$ ). Results of 3 independent experiments performed in quadruplicate are presented normalized to the result obtained in the absence of doxycycline taken equal to 1. Statistically significant variations are indicated by asterisks (\*,  $p < 0.05$ ).

of doxycycline were expected to represent different situations in which the entry of specific substrates into the peroxisome is altered. The induction of the WT ALDRP-EGFP protein was expected to boost the entry of some fatty acids and their subsequent degradation and then trigger a decrease in the membrane content for these substrates. On the contrary, induction of the D207H-ALDRP-EGFP protein was expected not to modify the fatty acid content of PL and serve as a control.

$\beta$ -Oxidation levels of  $^{14}\text{C}$ -labeled C26:0 and C24:0 were found increased in doxycycline-treated cells expressing the WT ALDRP-EGFP fusion protein. Moreover, we found an inverse correlation between the expression level of ALDRP-EGFP and the fatty acid content in PL of VLCFA (C26:0, C24:0, C26:1, and C24:1). These findings are consistent with the observed accumulation of C26:0 and C24:0 in tissues rich in ALDRP in *Abcd2* null mice (19, 29). Altogether, these results suggest a role of ALDRP in the transport of these fatty acids and are in agreement with the recognized partial functional redundancy between ALDP and ALDRP toward these substrates.

ALDRP has been suggested to play a role in the transport of other saturated and monounsaturated fatty acids such as C20:0, C22:0, and C22:1 (19). The putative role of ALDRP toward these fatty acids has been suggested from the observed accumulation of such fatty acids in specific tissues (adrenals and sciatic nerve) of *Abcd2* null mice (19). In our experiments, we could not evidence any changes in the content of these fatty acids. This apparent contradiction could be explained by the short time window (18 h) and by the fact that the doxycycline-dependent variations of ALDRP-EGFP expression are probably not sufficient to drastically change the overall catabolism of these fatty acids. Indeed, LCFA are known to be mainly oxidized in mitochondria. Moreover, our experiments were performed in hepatoma cell models. In the liver of *Abcd2* null mice, a modification of the level of these fatty acids was observed only after a 12-month specific diet (19). Altogether, our results do not evidence a role of ALDRP in the transport of saturated and mono-unsaturated LCFA but do not exclude such a role.

Concerning PUFA, the analysis of the *n*-3 and *n*-6 PUFA content in PL did not show any variation depending on the expression level of ALDRP-EGFP. However,  $\beta$ -oxidation assays with radiolabeled DHA showed that ALDRP is involved in the transport of DHA. Induction of ALDRP-EGFP resulted in a significant increase of the  $\beta$ -oxidation level of DHA. PUFA content in cells is highly regulated and depends on complex metabolic pathways. Its synthesis from C18:3*n*-3 requires elongation/desaturation steps and one final cycle of peroxisomal  $\beta$ -oxidation, which forms DHA from its immediate precursor C24:6*n*-3 (30, 31). DHA is also  $\beta$ -oxidized in the peroxisome. Although ALDP is clearly not involved in DHA metabolism because there is no deficiency in DHA in X-ALD patients, ALDRP-EGFP could participate in the entry of DHA. This would fit with the fact that the hepatic expression of ALDRP is highly sensitive toward dietary PUFA (32). Moreover ALDRP was recently suspected to participate in the entry of C24:6*n*-3 (the immediate precursor of DHA) into the peroxisome as suggested from *Abcd2* null mice (19). This dual involvement of ALDRP in the synthesis and degradation of DHA would explain in part the apparent discrepancy between the results from  $\beta$ -oxidation assays and GC-MS analysis in PL. Further explorations of the role of ALDRP in *n*-3 and *n*-6 PUFA metabolism are required but depend on the availability of radiolabeled fatty acids.

The cell clone expressing a mutated ALDRP-EGFP fusion protein was first used to validate the results obtained with the cell clone expressing the WT ALDRP-EGFP fusion protein. The direct correlation between the level of C26:0 and C26:1 in PL and the expression level of the D207H-ALDRP-EGFP fusion protein demonstrated a transdominant-negative effect of the mutant protein. This effect was also found in  $\beta$ -oxidation assays as a decreased  $\beta$ -oxidation activity of C26:0 was observed upon induction of D207H-ALDRP-EGFP. Duolink and coimmunoprecipitation experiments have evidenced interactions between D207H-ALDRP-EGFP and ALDP. The formation of a non-functional heterodimer D207H-ALDRP-EGFP/ALDP would deprive the cell of functional ALDP/ALDP homodimers and then result in the accumulation of C26:0 and C26:1 mimicking a deficiency on ALDP. Surprisingly, we did not observe

such a transdominant-negative effect on the ALDP function toward C24:0. The deprivation in functional ALDP/ALDP homodimers is maybe insufficient to see an accumulation in our cell model concerning this fatty acid. There are currently no clear data about differences in affinity toward C24:0 and C26:0 and/or transport efficiency, which could help to clarify this point. Altogether, we can conclude that D207H-ALDRP-EGFP has a transdominant-negative effect on ALDP function. This is the first demonstration that heterodimerization of peroxisomal half-transporters has functional consequences.

This transdominant-negative effect raises several points. The first one concerns therapeutic approaches aimed at overexpressing the *ABCD2* gene to compensate for ALDP deficiency. In the absence of the ALDP protein, this strategy remains pertinent. However, in patients in whom a non-functional ALDP is still present and capable to dimerize, pharmacological induction of the ALDRP expression would possibly result in the formation of a majority of non-functional heterodimers. Because a transdominant-negative effect of a mutated ALDP on ALDP function has already been demonstrated (25), one can speculate if restoring expression of ALDP in patients expressing a non-functional ALDP protein still able to dimerize would allow compensation for ALDP deficiency. Indeed, the successful results of gene therapy were recently obtained concerned two patients having an *ABCD1* gene mutation resulting in the absence of ALDP (33). A second point concerns the clear demonstration of the existence of heterodimers and their apparent specificity toward fatty acids substrates. Although the induction of the WT ALDRP-EGFP protein increases the  $\beta$ -oxidation of C26:0, C24:0, and C22:6*n*-3, and results in a decreased content of C26:0, C24:0, C26:1, and C24:1 in PL, the induction of the mutant ALDRP-EGFP protein has consequences only for C26:0 and C26:1. This would mean that ALDRP/ALDP heterodimers present a preferential substrate specificity toward C26:0 and C26:1, which would be different and more restricted than that of ALDP/ALDP or ALDRP/ALDRP homodimers. ALDP/ALDP homodimers would be dedicated to the transport of saturated and monounsaturated VLCFA (C26:0, C24:0, C26:1, and C24:1). ALDRP/ALDRP homodimers would be able to transport the same fatty acyl-CoA and be specifically dedicated to the transport of PUFA. During submission of this manuscript, functional complementation experiments in the yeast *pxa1/pxa2* $\Delta$  mutant with either *ABCD1* or *ABCD2* human genes were published (34). The main conclusions of this study conducted with a different experimental approach are in agreement with our conclusions. They confirmed the substrate specificity overlap between ALDP and ALDRP and demonstrated a preference of ALDP for saturated VLCFA (C24:0 and C26:0) and a preference of ALDRP for C22:0 and *n*-3 PUFA. In agreement with our results, a specific role of ALDRP in C22:6  $\beta$ -oxidation was evidenced.

Even if our results together with lessons from *Abcd1* and *Abcd2* null mice are in agreement with the different hypotheses concerning the substrate specificity of homo- and heterodimers, such a model of transport remains speculative. Further studies will be needed to clarify the role of each dimer and see if alternative dimerization depends only on relative stoichiometry or is driven by the substrates.

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