The mouse as a model to understand peroxisomal disorders

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CHAPTER 4

IMPAIRED NEURONAL MIGRATION AND ENDOCHONDRAL OSSIFICATION IN PEX7 KNOCKOUT MICE: A MODEL FOR RHIZOMELIC CHONDRODYSPLASIA PUNCTATA


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Impaired neuronal migration and endochondral ossification in Pex7 knockout mice: a model for rhizomelic chondrodysplasia punctata

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Rhizomelic chondrodysplasia punctata is a human autosomal recessive disorder characterized by skeletal, eye and brain abnormalities. The disorder is caused by mutations in the PEX7 gene, which encodes the receptor for a class of peroxisomal matrix enzymes. We describe the generation and characterization of a Pex7 knockout (Pex7−/−) mice. Pex7−/− mice are born severely hypotonic and have a growth impairment. Mortality in Pex7−/− mice is highest in the perinatal period although some Pex7−/− mice survived beyond 18 months. Biochemically Pex7−/− mice display the abnormalities related to a Pex7 deficiency, i.e. a severe depletion of plasmalogens, impaired α-oxidation of phytanic acid and impaired β-oxidation of very-long-chain fatty acids. In the intermediate zone of the developing cerebral cortex Pex7−/− mice have an increase in neuronal density. In vivo neuronal birthdating revealed that Pex7−/− mice have a delay in neuronal migration. Analysis of bone ossification in newborn Pex7−/− mice revealed a defect in ossification of distal bone elements of the limbs as well as parts of the skull and vertebrae. These findings demonstrate that Pex7 knockout mice provide an important model to study the role of peroxisomal functioning in the pathogenesis of the human disorder.

INTRODUCTION

Peroxisomes are ubiquitous cellular organelles, which participate in a variety of metabolic processes including the β-oxidation of very long-chain fatty acids (VLCFA) and fatty acid derivatives, α-oxidation of phytanic acid and the biosynthesis of ether-phospholipids (1). The peroxisomal proteins involved in these processes are synthesized on free cytosolic ribosomes and contain specific peroxisomal targeting signals (PTSS), which promote their targeting to the peroxisome (2).

Two types of PTSs have been identified in proteins destined for the peroxisomal matrix. PTS-type 1 (PTS1) is a carboxyl-terminal tripeptide following the consensus (SAC)(KRH)L (3,4), whereas PTS-type 2 (PTS2) comprises a nonapeptide sequence following the consensus (RK)(LVI)x5(HQ)L and resides within the amino-terminal region of the protein (5,6). In mammals and plants, the amino-terminal region containing the PTS2 sequence is cleaved off in the peroxisomal matrix (7). Following the recognition of PTS1 and PTS2 sequences by their cognate receptors peroxin 5 and peroxin 7, respectively, the
Rhizomelic chondrodysplasia punctata (RCDP) is an autosomal recessively inherited peroxisomal disorder characterized by a series of abnormalities including the presence of stippled foci of calcification within hyaline cartilage, dwarfism due to symmetrical shortening of proximal long bones (rhizomelia), congenital cataracts, hypotonia and severe psychomotor retardation (21,22). Prognosis is poor with death often occurring in early childhood. Phenotypic heterogeneity has been observed as patients have been described with mild manifestations of the disease with absence of rhizomelia and survival beyond the first decade of life (23–26). The genetic basis for RCDP type 1 (MIM 215100) lies in the PEX7 gene (11–13) in which more than twenty mutations have been identified (23,27,28). The product of the PEX7 gene, i.e. the protein Pex7, functions as the receptor that delivers newly synthesized cytosolic PTS2-containing proteins to peroxisomes. RCDP type 1 patients thus exhibit a specific disruption in PTS2-mediated protein import into peroxisomes. The biochemical abnormalities in RCDP type 1 are a reflection of this deficiency in protein import. So far, three bona-fide PTS2-dependent proteins have been identified: (1) alkyl-dihydroxyacetonephosphate synthase (alkyl-DHAP synthase), a key enzyme in ether-phospholipid biosynthesis (29); (2) phytanoyl-CoA hydroxylase (PHAX), involved in phytanic acid ß-oxidation (30–32); and (3) 3-oxoacyl-CoA thiolase (thiolase) (33). Owing to a defect in PTS2-mediated import, the biosynthesis of ether-phospholipids (plasmalogens) is deficient in patients with RCDP type 1 (34). Furthermore, there is an age- and diet-related accumulation of phytanic acid due to the deficient activity of PHAX resulting from its mistargeting (35). Although peroxisomal thiolase contains a PTS2-sequence and is mistargeted in RCDP type 1, no biochemical abnormality in straight chain fatty acid ß-oxidation has been observed in patients (21,36,37).

The importance of peroxisomal functioning for endochondral bone formation and eye and brain development, as inferred from the pathophysiology of PBDs and of RCDP, has remained elusive due to the lack of adequate model systems. Here we report the generation of a Pex7 knockout mouse (Pex7<sup>−/−</sup>). Biochemical analysis ascertained that Pex7<sup>−/−</sup> mice exhibit the abnormalities expected from a deficiency in Pex7, which are similar to those observed in RCDP type 1 patients. Phenotypic analysis indicates that Pex7<sup>−/−</sup> mice also display eye, brain and bone abnormalities mimicking those observed in RCDP type 1, thus making it a suitable animal model for this disorder.

RESULTS

Generation and phenotype of Pex7-deficient mice

The DNA construct for gene targeting was engineered to promote the disruption of Pex7 with the positional insertion of the E.coli ß-galactosidase reporter gene fused with the neomycin phosphotransferase gene (ß-geo cassette) (38), in the region of Pex7 exon 3 (Fig. 1A). Accordingly, G418 resistance is dependent on Pex7 promoter driven expression of the ß-geo cassette. Heterozygous disruption of the Pex7 gene was achieved by homologous recombination after electroporation of the targeting vector into R1 embryonic stem (ES) cells (Fig. 1B). Recombination events were identified in G418-resistant ES colonies at a frequency of 30%. One ES clone harboring a correct recombination event led to germline transmission. Pex7<sup>+/−</sup> mice were viable, fertile and not distinguishable from wild-type mice in all subsequent phenotypic analyses. Homozygous mutant mice (Pex7<sup>−/−</sup>) were obtained by intercrossing Pex7<sup>+/−</sup> mice. The genotype distribution of 136 individual newborn mice was in accordance with a Mendelian segregation of an autosomal recessive allele (35 +/-; 67 +/-; 34 +/-) indicating that lack of Pex7 does not result in embryonic lethality. Analysis of Pex7 transcripts by PCR amplification of cDNA, indicated that in Pex7<sup>−/−</sup> mice, mRNA from the targeted allele lacks exons located 3' to the insertion of the SA-IRES-ß-geo cassette (Fig. 1C).

Homozygous mutant mice were born alive, exhibited a variable degree of dwarfism and hypotonia with decreased motility, hampering them feeding. On the day of birth, Pex7<sup>−/−</sup> mice weighed 70–85% of their wild-type and heterozygous littermates. Approximately 50% of the Pex7<sup>−/−</sup> pups died on P0.5, 20% before weaning, and the remaining Pex7<sup>−/−</sup> mice survived beyond 18 months. The survival seemed to depend on the ability to overcome a critical period after birth. The cause of death in Pex7<sup>−/−</sup> mice remains unknown. Close monitoring of births revealed that Pex7<sup>−/−</sup> newborn pups were able to breathe, indicating normal lung development. Mutant mice reaching adulthood were not fertile and we observed testicular atrophy in Pex7<sup>−/−</sup> males (data not shown). Eye cataracts were invariably present in Pex7<sup>−/−</sup> mice (data not shown) and were first detected on the day mice opened their eyes.

Normal peroxisome assembly with impaired PTS2-mediated import

PTS-mediated protein import into peroxisomes was evaluated by immunofluorescence analysis in primary murine embryonic fibroblasts (MEFs) obtained from E16.5 wild-type and Pex7<sup>−/−</sup> embryos. A punctate localization pattern for catalase in both wild-type and Pex7<sup>−/−</sup> cell lines reveals that disruption of Pex7 does not interfere with PTS1-mediated protein import (Fig. 2A). The same results were obtained with antibodies against acyl-CoA oxidase and the SKL tripeptide (data not shown). The catalase-containing structures represent peroxisomes as concluded from the co-localization with the peroxisomal membrane marker, PMP70 (Fig. 2A).

The specific impairment of Pex7<sup>−/−</sup> mice to import PTS2-containing proteins was evaluated by immunoblot analysis. In protein extracts from liver and brain tissues of P0.5 Pex7<sup>−/−</sup>
mice, thiolase was present in its precursor form of 44 kDa, while in extracts from the same organs of wild-type mice thiolase was present in its mature form of 41 kDa (Fig. 2B). In extracts from P0.5 \( Pex7^{-/-} \) mice, alkyl-DHAP synthase was absent indicating that the precursor form is unstable in the cytosol and is prone to degradation (Fig. 2B). Alkyl-DHAP synthase was detected in its mature form in liver and brain extracts from P0.5 \( Pex7^{+/-} \) mice (Fig. 2B). In liver and brain extracts from \( Pex7^{-/-} \) mice, we found normal import and proteolytic processing of acyl-CoA oxidase as judged by the presence of both the 70 kDa precursor and the 50 kDa mature form (Fig. 2C).

**Pex7-related biochemical abnormalities**

The functional consequences of the observed defect in the import of PTS2-carrying proteins were substantiated by...
biochemical analyses of plasma, erythrocytes, and tissues. Firstly, plasmalogen levels in erythrocytes, fibroblasts and brain tissue were markedly reduced in Pex7\(^{-/-}\) mice, in contrast to Pex7\(^{+/+}\) and Pex7\(^{+/+}\) littermates (Table 1). De novo plasmalogen biosynthesis was determined in MEFs and in fibroblasts obtained from P0.5 pups (murine fibroblasts; MFs). In Pex7\(^{+/+}\) and Pex7\(^{+/+}\)-derived cells significant levels of plasmalogens were formed, whereas Pex7\(^{-/-}\) derived cells showed markedly reduced rates of plasmalogen formation (up to 85% in the PE pool and up to 91% in the PC pool, Table 2).

Figure 2. Import of peroxisomal proteins. (A) Localization of PMP70 and catalase in MEFs of wild-type (Pex7\(^{+/+}\)) and knockout (Pex7\(^{-/-}\)) mice, by indirect double-immunofluorescence using rabbit anti-PMP70 (a-PMP70) and mouse anti-catalase (a-catalase). As in wild-type-derived cells (upper panels), Pex7\(^{-/-}\)-derived cells (lower panels) show co-localization of catalase with PMP70, indicative that PTS1-mediated import is functional in Pex7\(^{-/-}\) mice. (B) Western blot analysis using antibodies against peroxisomal thiolase (a-thiolase) revealed that in liver and brain tissue of Pex7\(^{-/-}\) mice (\(-/-\)), thiolase is present in its precursor form (44 kDa; open arrowheads) whereas in extracts from wild-type mice (+/+ thiolase is detected in its mature form (41 kDa; closed arrowheads). Western blot analysis using an antibody against alkyl-DHAP synthase (a-alkyl-DHAP) revealed lack of detectable protein in protein extracts from Pex7\(^{-/-}\) mice. As expected, in liver and brain from wild-type mice alkyl-DHAP synthase was detected in its mature form (67 kDa; closed arrowheads). (C) Western blot analysis using antibodies against peroxisomal acyl-CoA oxidase-I (a-AOX-I) revealed that in wild-type and Pex7\(^{-/-}\) mice processing of matrix enzymes is normal. The 70 kDa precursor form of AOX-I (open arrowheads) is processed to the 50 kDa (closed arrowheads) and 20 kDa (not shown) mature forms.
showed a reduction of ~65% in the rate of C26:0 β-oxidation and ~59% in the rate of pristanic acid β-oxidation, when compared with Pex7+/− and Pex7+/+ fibroblasts (Fig. 3A). β-Oxidation of C16:0, which occurs predominantly in mitochondria, was not impaired (Fig. 3A). VLCFA levels in fibroblasts from Pex7−/− mice were also elevated (Fig. 3B), indicating that the disruption of thiolase import has functional consequences on peroxisomal β-oxidation. The levels of VLCFA in newborn pups confirmed that in Pex7−/− mice the disrupted import of thiolase leads to the accumulation of VLCFA in plasma, brain, and liver (Table 3). Surprisingly, 2-month-old Pex7−/− mice had normal levels of VLCFA in liver and brain (Table 3).

Levels of phytanic acid in plasma of P0.5 wild-type pups were undetectable, whereas Pex7+/− pups had low but measurable levels (1.7 ± 0.4 μmol/l, n = 6). The small accumulation of phytanic acid observed in newborn Pex7−/− mice is the consequence of deficient α-oxidation due to mistrargeting of PAHX. This was confirmed when a diet containing phytol was administered to 8-week-old wild-type and Pex7+/− mice. Supplementation of 0.5% free phytol to the standard chow for a period of 4 weeks led to a dramatic increase in phytic acid levels in plasma, liver and brain from Pex7−/− mice (Fig. 4). At the end of this dietary protocol Pex7−/− mice showed a 30% reduction of body weight whereas wild-type mice maintained their body weight.

**Impaired cortical neuronal migration**

In the developing brain, neuronal migration was evaluated at E18.5 by examining the layering of the neocortex in cresyl-violet-stained coronal sections. When compared to wild-type animals, Pex7−/− animals displayed an increased density of cells in the intermediate zone (Fig. 5A and C). However, this migration abnormality appeared to be less severe than that observed in Pex5−/− mice (Fig. 5B). More detailed analysis was done in E18.5 embryos by quantifying, in the intermediate zone (prospective white matter), neurons labeled with BrdU at E13.5. This technique confirmed that the intermediate zone of Pex7−/− mice was more densely populated with neurons as compared to wild-type mice (Fig. 5D), indicating that Pex7−/− mice have a defect in neuronal migration.

### Delayed endochondral ossification in Pex7−/− mice

Visual inspection of newborn pups did not reveal any abnormal patterning of hindlimbs or forelimbs in Pex7−/− newborn (P0.5) pups. Nevertheless, a more detailed analysis was pursued and skeletons of P0.5 mice were stained for bone and cartilage. This revealed that at birth, several cartilage-based structures were not ossified or showed a marked delay in ossification (Fig. 6). The skull of Pex7−/− pups had low but measurable levels (1.7 ± 0.4 μmol/l, n = 6). The small accumulation of phytanic acid observed in newborn Pex7−/− mice is the consequence of deficient α-oxidation due to mistrargeting of PAHX. This was confirmed when a diet containing phytol was administered to 8-week-old wild-type and Pex7+/− mice. Supplementation of 0.5% free phytol to the standard chow for a period of 4 weeks led to a dramatic increase in phytic acid levels in plasma, liver and brain from Pex7−/− mice (Fig. 4). At the end of this dietary protocol Pex7−/− mice showed a 30% reduction of body weight whereas wild-type mice maintained their body weight.

**Table 1. Plasmalogen levels**

<table>
<thead>
<tr>
<th></th>
<th>Erythrocytes</th>
<th>Brain</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DMAC16:C16:0</td>
<td></td>
</tr>
<tr>
<td>+/+ *</td>
<td>4.69 ± 0.80</td>
<td>13.15 ± 1.31</td>
</tr>
<tr>
<td>(n = 10)</td>
<td>6.18 ± 0.59</td>
<td>5.59 ± 0.12</td>
</tr>
<tr>
<td>−/− *</td>
<td>0.27 ± 0.23*</td>
<td>1.56 ± 0.93*</td>
</tr>
<tr>
<td>(n = 11)</td>
<td>0.1 ± 0.07*</td>
<td>0.66 ± 0.03*</td>
</tr>
</tbody>
</table>

The values (expressed as mean ± SEM) represent the percentage of the ratios between C16:0 or C18:0 dimethylacyl (DMA) derivatives of plasmalogens to their corresponding saturated fatty acids. Erythrocyte and brain samples were obtained from newborn wild-type and knockout mice. Fibroblasts were obtained from E16.5 and P0.5 wild-type and knockout mice. The number of samples used is shown in parentheses.

*aMice with PEX7+/− and PEX7−/− genotypes.

**Table 2. De novo plasmalogen biosynthesis in cultured fibroblasts**

<table>
<thead>
<tr>
<th></th>
<th>pPE/PE</th>
<th>pPC/PC</th>
<th>Ratio 14C/3H PE</th>
<th>Ratio 14C/3H PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ *</td>
<td>74 ± 5</td>
<td>11 ± 2</td>
<td>361 ± 56</td>
<td>717 ± 58</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−/− *</td>
<td>11 ± 6*</td>
<td>0.9 ± 0.2*</td>
<td>8 ± 5*</td>
<td>39 ± 12*</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td></td>
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</tbody>
</table>

The rate of plasmalogen biosynthesis is expressed as mean ± SEM of: pPE/PE [percentage of the ratio plasmalogenethanolamine (pPE) to phosphoethanolamine (PE)]; ratio 14C/3H PE (the ratio of radioactivity incorporated into PE from 3H-labeled alkylglycerol and 14C-labeled hexadecanol); pPC/PC [percentage of the ratio plasmalogencholine (pPC) to phosphocholine (PC)]; ratio 14C/3H PC (the ratio of radioactivity incorporated into PC from 3H-labeled alkylglycerol and 14C-labeled hexadecanol). Fibroblasts were obtained from E16.5 and P0.5 wild-type and knockout mice. The number of samples used is shown in parentheses.

*a Mice with PEX7+/− and PEX7−/− genotypes.

**Student’s t-test, P < 0.0001.**
Table 3. Very-long-chain fatty acid measurements in newborn and 2-month-old mice

<table>
<thead>
<tr>
<th>Age/sample</th>
<th>Genotype</th>
<th>Concentration of fatty acids&quot;</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C22:0</td>
<td>C24:0</td>
</tr>
<tr>
<td>P0.5 (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>+/-</td>
<td>10.92 ± 1.93</td>
<td>8.26 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>7.53 ± 0.68*</td>
<td>9.91 ± 1.87</td>
</tr>
<tr>
<td>Liver</td>
<td>+/-</td>
<td>0.78 ± 0.05</td>
<td>1.34 ± 0.15</td>
</tr>
<tr>
<td>Brain</td>
<td>+/-</td>
<td>0.68 ± 0.07</td>
<td>1.76 ± 0.19*</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>0.44 ± 0.05</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>+/-</td>
<td>0.43 ± 0.05</td>
<td>0.78 ± 0.07*</td>
</tr>
</tbody>
</table>

| P64.5 (n = 3) |          |       |       |       |           |           |
| Plasma       | +/-      | 11 ± 0.25 | 7.33 ± 0.45 | 0.01 ± 0.001 | 0.67 ± 0.06 | 0.001 ± 0.001 |
| Liver        | +/-      | 7.39 ± 3.26 | 8.90 ± 3.79 | 0.42 ± 0.15* | 1.21 ± 0.01* | 0.06 ± 0.004* |
| Brain        | +/-      | 1.91 ± 0.08 | 1.52 ± 0.05 | 0.1 ± 0.02 | 0.8 ± 0.02 | 0.03 ± 0.03 |
|            | +/-      | 0.99 ± 0.2* | 1.34 ± 0.21 | 0.11 ± 0.01 | 1.36 ± 0.1* | 0.12 ± 0.03* |
|            | +/-      | 2.29 ± 0.53 | 3.46 ± 0.63 | 0.13 ± 0.01 | 1.51 ± 0.09 | 0.04 ± 0.04 |
|            | +/-      | 1.74 ± 0.95 | 2.72 ± 1.53 | 0.16 ± 0.02 | 1.53 ± 0.05 | 0.11 ± 0.06 |

Values represent means ± SEM; *P < 0.05 (comparison to controls with Student's t-test).

"Concentration of fatty acids are expressed in μmol/L for plasma and μmol/mg protein for liver and brain.

(Fig. 6I and J). Size-matched Pex7<sup>+/−</sup> P0.5 pups did not reveal any ossification abnormalities similar to the ones present in Pex7<sup>−/−</sup> mice. Ossification of clavicle, calvaria and regions of the mandible were normal and did not reveal any delay in ossification (data not shown), suggesting that intramembranous ossification is not affected in Pex7<sup>−/−</sup> mice.

Histological analysis of femurs from Pex7<sup>−/−</sup> E18.5 embryos revealed a lower level of mineralization (data not shown). At the ultrastructural level the appearance of osteoblasts, chondrocytes and osteocytes was similar between wild-type and Pex7<sup>−/−</sup> mice. Osteoclasts were frequently noted in bones of both wild-type and Pex7<sup>−/−</sup> mice. The number and overall appearance was similar. In osteoclasts a clear zone and ruffled border were apparent, thus indicating that osteoclastic bone resorption occurred in wild-type and Pex7<sup>−/−</sup> mice. There was, however, one remarkable difference. Osteoclasts from Pex7<sup>−/−</sup> mice contained higher numbers of electron-translucent vacuoles (Fig. 7). These vacuoles were seen in close vicinity to the ruffled border of the cells. The majority of these vacuoles did not contain any recognizable structures and seemed empty, although in some vacuoles the presence of small crystal-like structures was noted (Fig. 7C and D).

DISCUSSION

RCDP type 1 is of special interest amongst the disorders caused by deficient peroxisomal functioning as only a subset of peroxisomal functions is impaired. Clinically, RCDP type 1 patients display a severe growth delay, cataracts, rhizomelia and epiphyseal calcifications. The defective gene in RCDP type-1, PEX7, encodes the receptor responsible for import of PTS2-containing proteins to peroxisomes. We have created a mouse model for RCDP type 1 by gene targeting in the murine Pex7 locus. The deficiency in PTS2-mediated import in Pex7<sup>−/−</sup> mice leads to biochemical abnormalities in: (1) the biosynthesis of plasmalogens; (2) the α-oxidation of phytanic acid; and (3) β-oxidation of VLCFA.

Plasmalogens are a class of ether-phospholipids with an α,β-unsaturated ether-bond at the sn-1 position of the glycerol backbone. Aside from serving as structural components of cellular membranes (39), plasmalogens have been implicated in the protection against oxygen reactive species (40), intracellular signaling (41) and the maintenance of high levels of polyunsaturated fatty acids in tissues (42–44). However, convincing evidence concerning their function is lacking. The present mouse model exhibiting a severe depletion in plasmalogens should be instructive to unravel the specific role of plasmalogens in cellular functioning, chondrocyte development and in the neuronal migration process.

Phytanic acid and phytol are not synthesized de novo by mammals and their concentrations in plasma and tissues depend on dietary intake (45). Regular mouse chow contains very small amounts of phytic acid or its precursors (46), explaining the low levels of phytic acid in plasma and tissues of Pex7<sup>−/−</sup> mice fed a normal diet. However, supplementing phytol to the diet resulted in a massive accumulation of phytic acid in Pex7<sup>−/−</sup> mice whereas phytic acid levels remained 10-fold lower in wild-type mice.

The third metabolic abnormality found in Pex7<sup>−/−</sup> mice was an impairment in the β-oxidation of VLCFA. In Pex7<sup>−/−</sup>-derived fibroblasts we found a reduction in C26:0 and pristanic acid β-oxidation rates, which was accompanied by an accumulation of C26:0 in the same cell lines. Measurements of VLCFA levels in plasma and tissues of newborn Pex7<sup>−/−</sup> mice revealed that the reduction in peroxisomal β-oxidation rates leads to an accumulation of VLCFA. Adult Pex7<sup>−/−</sup> mice had normal levels of VLCFA in tissues, indicating that with development the deficiency is overcome. The defect in peroxisomal β-oxidation is in contrast to the results found in RCDP type 1 patients. RCDP type 1 patients display normal β-oxidation of VLCFA and normal values of C26:0 in plasma despite the disruption in import of peroxisomal thiolase (47). Nevertheless it has been observed that erythrocytes, lymphoblasts and platelets of RCDP type 1 patients accumulate VLCFA to levels comparable to that of Zellweger and X-linked adrenoleukodystrophy patients (47). Several hypotheses have...
been proposed in order to explain the apparent contradiction between mistargeting of thiolase and normal $\beta$-oxidation rates. One hypothesis is that sterol carrier protein x (SCPx), which possesses a domain with thiolytic activity, takes over the function of peroxisomal thiolase and is responsible for the normal rates of C26:0 $\beta$-oxidation in RCDP type 1 (37,48). Other hypothetical scenarios include the import into peroxisomes of undetectable levels of thiolase or the shuttling of $\beta$-oxidation intermediary byproducts from the peroxisomal matrix to the cytosol, where the enzymatically active precursor form of thiolase would break them down for further $\beta$-oxidation in mitochondria. The finding in $Pex7^{-/-}$ mice that the defect in VLCFA $\beta$-oxidation is only detected in the prenatal and early postnatal period suggests that, if there is a mechanism to restore $\beta$-oxidation, it is dependent on age. It has been observed that in mice SCPx expression increases during development reaching high levels at 6 months of age (49). Therefore we favor the hypothesis that in $Pex7^{-/-}$ mice the defect in $\beta$-oxidation may be rescued by expression of SCPx. Accordingly, this hypothesis may also explain the RCDP scenario: in RCDP-derived cells that express SCPx normal $\beta$-oxidation rates are found and in

Figure 3. Impaired VLCFA $\beta$-oxidation in $Pex7^{-/-}$-derived fibroblasts. Black columns represent the mean values obtained with wild-type fibroblasts ($Pex7^{+/+}$ and $Pex7^{+/-}; n=6$) and gray columns represent the mean values obtained with $Pex7^{-/-}$ fibroblasts ($n=7$). Brackets represent the standard deviation of the mean. (A) $\beta$-Oxidation rates of C16:0, C26:0 and pristanic acid revealed that, when compared with wild-type-derived fibroblasts, $Pex7^{-/-}$ fibroblasts have a deficiency in peroxisomal VLCFA $\beta$-oxidation. A reduction of 65 and 59% in the rate of C26:0 and pristanic acid, respectively, is observed in $Pex7^{-/-}$-derived fibroblasts. In $Pex7^{-/-}$-derived fibroblasts the rate of C16:0 $\beta$-oxidation is similar to that of wild-type fibroblasts. (B) The levels of VLCFA (C24:0 and C26:0) are elevated in $Pex7^{-/-}$-derived fibroblasts when compared to wild-type fibroblasts. *$P=0.001$ with Student’s $t$-test.

Figure 4. Impaired phytol catabolism. Eight-week-old wild-type and $Pex7^{-/-}$ mice were fed a diet containing 0.5% free phytol for a period of 4 weeks (wild-type mice, $n=3$; $Pex7^{-/-}$ mice, $n=3$). The levels of phytanic acid were measured in plasma (A), liver (B) and brain (C) tissue. In plasma and tissues from $Pex7^{-/-}$ mice a drastic accumulation of phytanic acid is observed. Under a normal diet phytanic acid levels in wild-type and $Pex7^{-/-}$ mice-derived plasma and tissues are undetectable. Column nomenclature is identical to that in Figure 3. *$P<0.01$ with Student’s $t$-test.
cells that do not express or express low levels of SCPx accumulation of VLCFA occurs.

In Pex7−/− mice we found a disruption in the process of neuronal migration evident by the increased number of neuronal precursors in the intermediate zone of Pex7−/− developing neocortex. Stratification of the cerebral cortex is achieved with radial migration of postmitotic neurons formed in the subventricular zone towards the pial surface. The layering of cortical neurons follows an inside-out distribution in the subventricular zone towards the pial surface. The typical skeletal features of RCDP patients such as rhizomelia and the coronal clefing of vertebral bodies (66) were not apparent in Pex7−/− mice but the nature of these defects suggests a delay in the process of ossification similar to the one found in Pex7−/− mice. The sequential and synchronized differentiation of chondrocytes is tightly controlled during development. Extracellular ligands, their receptors, and various downstream cytoplasmic and nuclear transducers regulate the processes of chondrocyte maturation, differentiation, and hypertrophy. Molecules like retinoids (67,68) and vitamin D (69,70) as well as extracellular matrix proteins such as parathyroid-hormone-related peptide (71) and bone morphogenetic protein 6 (72,73) have been implicated in the switch to the hypertrophic stage of chondrocyte development. In Pex7−/− mice, the lack of plasmalogens in all cellular membranes may affect the signaling pathways essential to transduce both extracellular and intracellular signals required to regulate the final stages of chondrocyte development.
Figure 6. Impaired ossification in Pex7\textsuperscript{−/−} mice. Whole mount skeletal staining of cartilage (blue) and bone (red) of wild-type (Pex7\textsuperscript{+/+}; left column) and knockout (Pex7\textsuperscript{−/−}; right column) P0.5 pups. (A and B) View of the skull floor showing impaired ossification of the basi-phenoid bone (black arrows) of Pex7\textsuperscript{−/−} mice. (C and D) View of the skull floor showing impaired formation of ossification centers in the middle ear (white arrows) of Pex7\textsuperscript{−/−} mice. (E and F) Impaired formation of ossification centers in the transverse process of coccygeal vertebrae of Pex7\textsuperscript{−/−} mice (black arrowheads). (G and H) Delayed ossification of the calcaneus and lack of ossification in the talus of Pex7\textsuperscript{−/−} hindpaws (black arrows). (I and J) Lack of ossification in the middle phalanges (black arrows) of Pex7\textsuperscript{−/−} hindpaws. In (J) note the persistence of dense cartilage in the middle region of the phalange.
containing exons 1 and 2 comprises the 5′ HindIII subclone of the P1 clone) was cloned as the 3′ fragment was inserted. This SA-IRES-geo fragment contains a splice acceptor (SA) placed upstream of the polyadenylation signal] (75,76). Finally, an 8 kb XhoI fragment containing exons 1–3 of murine Pex7 was cloned in the forward orientation from the Pex7 gene [which comprises exons 1 and 2 comprises the 5′ flank of the targeting construct. The 250 bp probe was obtained by PCR with primers 5′-ACATGGAGGTTGCCATTTAAGCG-3′ (forward) and 5′-CGAGGTAGAAGTGGCCACTG-3′ (reverse; this primer anneals directly upstream of the XhoI site of the 5′ flank, which is 50 nucleotides upstream of the start codon). In the case of correct targeting a 3 kb band hybridized in addition to the 8 kb endogenous Pex7 band.

Pex7 clones showing a targeted band with the 5′ probe were checked for recombination events on the 3′ side by probing BamHI blots with a 3′ probe. This probe anneals in the 3′ flank, and is a 700 bp probe generated by PCR on the P1 Pex7 clone template using the primer set: forward primer 5′-CACGCTATGGTAGGGATGTG-3′ and reverse primer 5′-CTGGTTTGGCCTCAAATCAAC-3′. In the event of correct targeting, the BamHI site on the 3′ side is restored, resulting in an 8.5 kb targeted band in addition to the 12 kb endogenous band.

For analysis of Pex7 transcripts, total RNA was isolated from liver samples of wild-type and knockout mice using the Wizard RNA purification kit (Promega) and first-strand cDNA was prepared as previously described (77). cDNA was amplified by PCR using primer sets encompassing exons 1–5 (forward 5′-TTTCTTCCCGTACCTGCCG-3′ and reverse 5′-TGATAATGAACACTCTCATGGCC-3′) and exons 5–8 (forward 5′-TCCACACTGTGGAAATTTCTCTG-3′ and reverse 5′-GAGCGGTGGAACGGTAAA-3′). As a control for cDNA amplification we used primers against β-actin (78).

### Immunofluorescence and western blot analysis

Mouse embryonic fibroblasts (MEFs) were isolated from E16.5 embryos and mouse fibroblasts (MFs) were isolated from P0.5 pups as described (62). Fibroblasts were cultured in DMEM (Gibco, Invitrogen) supplemented with 20% fetal calf serum (Bio-Whittaker), 100 μ/ml penicillin, 100 μg/ml Streptomycin and 25 mM HEPES buffer, in a humidified atmosphere of 5% CO2 at 37°C. Indirect immunofluorescence was performed as described (79). The polyclonal antibody against PMP70 was purchased from Zymed and the mouse monoclonal against catalase was obtained from the supernatant of hybridoma 17E10 (80).

For western blot analysis, liver and brain tissue were homogenized in phosphate buffered saline (PBS) with 0.5% Triton X-100 and a cocktail of protease inhibitors (Roche). Thirty micrograms of protein was separated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted. Polyclonal antibodies against peroxisomal thiolase and acyl-CoA oxidase and alkyl-DHAP-synthase were used as described (81,82).
Biochemical assays

All biochemical assays were performed as previously described: de novo plasmalogens biosynthesis (83), β-oxidation in cultured fibroblasts (84), analyses of VLCFA (47) and phytanic acid (85) and plasmalogens (86). Brain, liver and fibroblasts were homogenized in PBS and 400 μg of protein were used in the assays for measurement of VLCFA, plasmalogens and phytanic acid.

Histology

For routine histological analysis, brains of E18.5 pups were fixed in 4% paraformaldehyde and embedded in paraffin. Seven micrometer-thick coronal sections were used in the assays for immunohistochemical detection of BrdU. Based on previous studies performed in Pex5 knockout mice (55,87), we used the density of BrdU-stained cells as an index of the severity of the neuronal migration disorder. To avoid regional and experimental variations in labeling, sections from the different experimental groups, including comparable anatomical regions in the frontoparietal area (SI), were treated simultaneously. Counts of BrdU-positive cells were performed in a 0.025 mm² area of the intermediate zone (prospective white matter); for each experimental group, cells were counted in 10 different fields (five brains from three different litters, two non-adjacent sections of the right hemisphere per brain).

Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Leuven. The diet enriched with 0.5% phytol (Acros) was prepared by dissolving 2.5 g phytol in 35 ml acetone, distributing it evenly on 500 g of pelleted mouse food followed by drying overnight.

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