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Genetic classification and mutational spectrum of more than 600 patients with a Zellweger syndrome spectrum disorder

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2 **Genetic classification and mutational spectrum of more than 600 patients with a**
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4 **Zellweger syndrome spectrum disorder**

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Abstract

The autosomal recessive Zellweger syndrome spectrum (ZSS) disorders comprise a main subgroup of the peroxisome biogenesis disorders and can be caused by mutations in any of 12 different currently identified *PEX* genes resulting in severe multi-systemic disorders. To get insight into the spectrum of *PEX* gene defects among ZSS disorders and to investigate if additional human *PEX* genes are required for functional peroxisome biogenesis, we assigned over 600 ZSS fibroblast cell lines to different genetic complementation groups. These fibroblast cell lines were subjected to a complementation assay involving fusion by means of polyethylene glycol or a *PEX* cDNA transfection assay specifically developed for this purpose. In a majority of the cell lines we subsequently determined the underlying mutations by sequence analysis of the implicated *PEX* genes. The *PEX* cDNA transfection assay allows for the rapid identification of *PEX* genes defective in ZSS patients. The assignment of over 600 fibroblast cell lines to different genetic complementation groups provides the most comprehensive and representative overview of the frequency distribution of the different *PEX* gene defects. We did not identify any novel genetic complementation group, suggesting that all *PEX* gene defects resulting in peroxisome deficiency are currently known.

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Objective:Deleted: ¶
Methods: FDeleted: ¶
Results and Discussion:**Key words**

Zellweger Syndrome, *PEX* genes, genetic complementation, peroxisome, biogenesis

Introduction

Peroxisome biogenesis disorders (MIM 601539) include the Zellweger syndrome spectrum (ZSS) disorders and Rhizomelic Chondrodysplasia Punctata type I (Weller et al., 2003). The latter is caused by mutations in the *PEX7* gene, encoding a cytosolic receptor involved in the import of a small subset of peroxisomal matrix proteins (Braverman et al., 2002; Motley et al., 2002). The ZSS disorders include the Zellweger syndrome (ZS, MIM 214100), neonatal adrenoleukodystrophy (NALD, MIM 202370) and infantile Refsum disease (IRD, MIM 266510), which represent a spectrum of disease severity with ZS being the most, and IRD the least severe disorder.

ZSS disorders are autosomal recessive disorders and can be caused by a defect in any of at least 12 different *PEX* genes (Steinberg et al., 2006). These *PEX* genes encode proteins called peroxins that are involved in various stages of peroxisomal protein import and/or the biogenesis of peroxisomes. Severe defects in one of these *PEX* genes result in a complete absence of functional peroxisomes as observed in the Zellweger Syndrome phenotype. The NALD or IRD phenotypes are often associated with residual peroxisomal functions and/or partial functional peroxisomes (i.e. peroxisomal mosaicism (Wanders and Waterham, 2006; Weller et al., 2003)).

Many human *PEX* genes have been identified on the basis of sequence similarity with yeast *PEX* genes identified by functional complementation of peroxisome-deficient yeast mutants. At present more than 30 different peroxins have been identified in different yeast species, including *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. The peroxins are involved in different peroxisomal processes, such as the formation of peroxisomal membranes, peroxisomal growth, fission and proliferation, and import of matrix proteins (Platta and Erdmann, 2007). Of these yeast peroxins, 19 have been implicated in peroxisome protein import and biogenesis, whereas in humans, so far only 13 peroxins have been

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2 identified that are required for these processes. To study whether human peroxisome
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4 biogenesis requires the action of additional peroxins, we set out to assign primary skin
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6 fibroblasts from patients diagnosed with a ZSS disorder to different genetic complementation
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8 groups. Following the complementation analysis, we performed mutation analysis of the
9
10 respective *PEX* genes in a majority of cell lines. We here report the results of our genetic
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12 complementation studies in more than 600 cell lines from patients with a Zellweger syndrome
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14 spectrum disorder. In addition, we provide an overview of all mutations we identified in these
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16 patient cell lines, completed with previously reported mutations in the respective *PEX* genes.
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For Peer Review

Methods

Cell lines

For this study, we used 613 independent primary skin fibroblast cell lines from non-related patients that had been sent to our laboratory since 1985 for diagnostic evaluation and which were diagnosed with a ZSS disorder. The diagnosis is based on metabolite analysis in plasma, which ideally involves analysis of very long chain fatty acids (VLCFAs), bile acid intermediates, phytanic acid, pristanic acid, pipelicolic acid and plasmalogens, and/or detailed studies in fibroblasts, including VLCFA analysis, C26:0 and pristanic acid β -oxidation, phytanic acid α -oxidation and [dihydroxyacetonephosphate acyltransferase \(DHAPAT\)](#) activity analysis, and the absence of peroxisomes assessed by on catalase immunofluorescence (IF) microscopy (Wanders and Waterham, 2005). The cell lines were cultured in DMEM medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Bio-Whittaker), 100 U/ml penicillin, 100 μ g/ml streptomycin and 25 mM Hepes buffer with L-glutamine in a humidified atmosphere of 5% CO₂ and at 37°C. In accordance to the institutional guidelines and the Dutch Code of Conduct, identifiable clinical and personal data from the patients were not available for this study.

Genetic complementation assays

To assign cell lines to genetic complementation groups, we used a previously described polyethylene glycol (PEG)- mediated cell fusion assay (Brul et al., 1988) and a *PEX* cDNA transfection assay, specifically developed for this purpose. In the latter assay, ZSS cell lines are co-transfected separately with [one of the 12](#) different *PEX* cDNAs subcloned in the pcDNA3 expression vector ([Invitrogen, Carlsbad, CA](#)) and an eGFP-SKL vector (Waterham et al., 2007). The eGFP-SKL vector expresses eGFP tagged with the peroxisomal targeting signal -SKL, which allows immediate visual detection of peroxisomes when present. The

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2 transfections were performed with the NHDF Nucleofector Kit (Amaxa, Cologne, Germany)
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4 and the Nucleofector Device (Amaxa, Cologne, Germany) using the standard program U-23,
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6 which in our hands results in high transfection efficiencies ranging from 70-80% for primary
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8 skin fibroblasts. We performed the transfections of cell lines in three subsequent series based
9
10 on the estimated frequency of the different PEX gene defects. In the first series, we
11
12 transfected cell lines separately with PEX1, PEX6 and PEX12. Cell lines that were not
13
14 functionally complemented were then transfected separately with PEX2, PEX10 and PEX26
15
16 and finally with PEX5, PEX13 and PEX14. Transfection with PEX3, PEX16 and PEX19 was
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18 performed when the patient fibroblasts showed no peroxisomal membrane remnants based on
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20 immunofluorescence microscopy (van Grunsven et al., 1999) with antibodies against ALDP
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22 (Euromedex, France, Souffelweyersheim), a peroxisomal membrane protein. Cells were
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24 examined by means of fluorescence microscopy 48 to 72 hours after transfection to determine
25
26 the subcellular localization of the peroxisomal reporter protein eGFP-SKL (see also figure 1).
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Deleted: used the following transfection order of PEX expression vectors based on the estimated frequency of the different PEX gene defects: 1)

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30 Mutation analysis

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32 Mutation analysis was performed by either sequencing all exons plus flanking intronic
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34 sequences of the PEX genes amplified by PCR from genomic DNA or by sequencing PEX
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36 cDNAs prepared from total RNA fractions. Genomic DNA was isolated from skin fibroblasts
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38 using the NucleoSpin Tissue genomic DNA purification kit (Macherey-Nagel, Germany,
39
40 Düren). Total RNA was isolated from skin fibroblasts using Trizol (Invitrogen, Carlsbad, CA)
41
42 extraction, after which cDNA was prepared using a first strand cDNA synthesis kit for RT-
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44 PCR (Roche, Mannheim, Germany). All forward and reverse primers (Supp tables S1-11)
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46 were tagged with a -21M13 (5'-TGTAACGACGGCCAGT-3') sequence or M13rev (5'-
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48 CAGGAAACAGCTATGACC-3') sequence, respectively. PCR fragments were sequenced in
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50 two directions using '-21M13' and 'M13rev' primers by means of BigDye Terminator v1.1
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2 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and analyzed on an
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4 Applied Biosystems 377A automated DNA sequencer, following the manufacturer's protocol
5
6 (Applied Biosystems, Foster City, CA, USA).
7

8 Mutation analysis of *PEX1* (GenBank accession number NM_000466.2) was performed by
9 sequencing the coding region of the *PEX1* cDNA and/ or exons amplified from gDNA or by
10 RFLP analysis to identify the frequently occurring c.2528G>A mutation. The RFLP analysis
11 involves PCR amplification using a specific primer set (see Supp Table S1) of a 130-bp
12 genomic DNA fragment encompassing the c.2528G/A nucleotide, followed by restriction
13 analysis with *ClaI*. The c.2528G>A mutation creates a restriction recognition site for *ClaI* and
14 thus can be screened for by RFLP analysis. Mutations in the *PEX2* gene were identified by
15 sequencing the coding exon 4 from gDNA (NM_000318.2). The *PEX3* (NM_003630.2) gene
16 was analyzed by sequencing of the coding region of the *PEX3* cDNA. Mutation analysis of
17 *PEX5L* (X84899), *PEX10* (NM_002617.3), *PEX12* (NM_000286.2), *PEX13* (NM_002618.2),
18 *PEX14* (NM_004565.2), *PEX16* (NM_004813.2), *PEX19* (NM_002857.2) and *PEX26*
19 (NM_017929.4) were performed by sequencing of all exons plus flanking intronic sequences
20 amplified from gDNA. Sequences were compared with the indicated reference sequences with
21 nucleotide numbering starting at the first adenine of the translation initiation codon ATG. The
22 possible consequences of missense mutations were analyzed using the Polyphen prediction
23 software (<http://genetics.bwh.harvard.edu/pph/>)
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Results and Discussion

Genetic complementation

Initially, we used a previously reported cell fusion complementation assay (Brul et al., 1988). Because this assay is rather laborious, we developed a more rapid and direct complementation assay taking advantage of the known identities of the 12 human *PEX* genes that can cause a ZSS disorder (for principle see figure 1). The *PEX* cDNA transfection assay worked very well for cell lines displaying complete peroxisome deficiencies, and for cell lines displaying different degrees of peroxisomal mosaicism that are sensitive to culturing at 40°C, which exacerbates the defect in peroxisome biogenesis (Gootjes et al., 2004b). We assigned 613 different ZSS cell lines to genetic complementation groups (Table 1). The results give a representative overview of the spectrum of *PEX* gene defects among ZSS disorders with *PEX1* (MIM602136), *PEX6* (MIM601498) and *PEX12* (MIM601758) defects being the most common. Although we received ZSS cell lines from different parts of the world, they predominantly (>90%) are from Western European origin and thus the spectrum of *PEX* gene defects primarily relates to the Western European population.

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Somewhat unexpected, all the analyzed ZSS cell lines could be assigned to one of the known genetic complementation groups and no novel complementation group was identified. In a recent study where 90 cell lines were assigned to genetic complementation groups using a similar approach also no novel complementation groups were identified (Krause et al., 2009). This suggests that, in contrast to yeast where 19 different *PEX* genes have been implicated, peroxisome biogenesis in humans would only require 12 *PEX* genes. However, it should be emphasized that to be suitable for genetic complementation testing, cell lines need to display a complete peroxisome deficiency in the majority of cells. It therefore remains possible that defects in other putative human *PEX* genes cannot be identified by this approach, because they don't result in peroxisome deficiency or perhaps lead to different clinical presentations,

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1
2 as recently exemplified by a very distinct peroxisomal and mitochondrial fission defect due to
3
4 a dominant negative mutation in the *DLP1* gene (Waterham et al., 2007). In addition, defects
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6 in certain *PEX* genes may also be without consequences due to gene redundancy. For
7
8 example, recently 3 human proteins have been reported with a similar function as the yeast
9
10 *PEX4* gene, which is associated with complete peroxisome deficiency in yeast (Grou et al.,
11
12 2008). Considering that these proteins are encoded by different genes and each can promote
13
14 the ubiquitination of *PEX5* (MIM600414) on its own, it seems unlikely that a defect in one of
15
16 these proteins will cause a peroxisomal disorder (Grou et al., 2008). Finally, although we
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18 consider this unlikely, we cannot exclude the possibility that a defect in certain unannotated
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20 *PEX* genes may be embryonic lethal and thus will not be diagnosed.

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21 22 23 24 *Mutation analysis*

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26 After the assignment of patient cell lines to genetic complementation groups, we performed
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28 mutation analysis of the respective *PEX* genes in 393 of the cell lines. For most genes all
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30 coding exons plus flanking intron sequences were analysed by sequencing. In principle, this
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32 method will identify all missense and nonsense mutations, small deletions, small duplications
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34 and small insertions located in the analysed fragments. However, the presence of large,
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36 heterozygous deletions or duplications, or pathogenic mutations located outside the analysed
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38 fragments cannot be excluded. The reported mutations are assumed to be pathogenic for the
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40 following reasons: 1) They are the only sequence variants identified in the coding regions or
41
42 flanking intronic sequences of a given *PEX* gene that was demonstrated to be defective by the
43
44 fact that peroxisome biogenesis in the corresponding cell line is restored after transfection
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46 with the respective *PEX* cDNA (i.e. a functional assay). 2) The sequence variants are not
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48 listed in the SNP database or encountered in more than 100 control chromosomes.
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PEX1 is the most affected gene among patients with a ZSS disorder. It encodes a protein belonging to the AAA ATPase protein family (ATPases Associated with various cellular Activities) and contains two ATP-binding folds. Table 2 lists all mutations identified in the cell lines we analyzed completed with mutations reported in literature. Among these mutations a few common mutations have been identified (Maxwell et al., 2005). The most common mutation in *PEX1* is a missense mutation in the second ATP-binding domain (c.2528G>A) leading to p.G843D. This mutation reduces the binding between PEX1 and PEX6 and is known for its temperature sensitivity, meaning that when fibroblasts of patients homozygous for G843D are cultured at 30°C, they regain the ability to import catalase and other peroxisomal matrix proteins, as well as the various peroxisomal metabolic functions (Imamura et al., 1998a; Maxwell et al., 2005). Patients homozygous for the G843D mutation usually present with the milder IRD phenotype. Cells of these patients often display peroxisomal mosaicism at 37°C, but a complete peroxisome deficiency when cultured at 40°C. The second most common mutation in *PEX1* is c.2079_2098insT, which results in a frame shift predicted to cause a truncated PEX1 protein (Collins and Gould, 1999), although Northern blot analysis showed that this mutation results in low steady state *PEX1* mRNA levels (Maxwell et al., 2005). Patients homozygous for this mutation are affected with the severe ZS phenotype. The c.2926delA mutation is the third most common mutation in *PEX1*; this mutation leads to a frame shift. When combined with previously reported *PEX1* mutations, a total of 88 different mutations have now been identified in the *PEX1* gene, of which 34 have not been reported previously. In two cell lines we found three heterozygous mutations after sequencing gDNA (Table 1). In the first cell line the following 3 heterozygous mutations were found, p.T526A, p.911_912delCT and p.1885_1886delGT, of which the last two have been reported as pathogenic in literature. The consequence of the p.T526A mutation is unclear, but the threonine at position is a conserved amino acid in *Pan troglodytes*

Deleted: It should be noted that in the vast majority of cell lines we identified either two heterozygous or one homozygous putative pathogenic mutation, in few cell lines we only found one heterozygous mutation after sequencing gDNA, which in most cases appeared homozygously expressed when analyzing the respective *PEX* cDNA, indicating that the second, unidentified mutation affects gene transcription of mRNA stability. ¶

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2 (chimpanzee), *Canis familiaris* (dog) and *Gallus gallus domesticus* (chicken) and the software
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4 program Polyphen predicts this amino acid substitution as possibly damaging. In the second
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6 cell line, the heterozygous p.V336A, p.S555P and p.R949W missense mutations all affect
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8 well conserved amino acids and, according to Polyphen, are predicted to be benign, possibly
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10 damaging and probably damaging, respectively, suggesting that p.V336A may be a rare
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12 sequence variation. All the novel identified missense mutations are not listed in the SNP
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14 database, have not been detected in more than 100 control chromosomes and in fact all affect
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16 (well) conserved amino acids. In addition, Polyphen predicts these amino acid substitutions as
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18 either possibly damaging or probably damaging.

19
20 The *PEX2* (MIM170993) gene was the first gene reported to be mutated in ZSS
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22 patients. *PEX2* is an integral membrane protein with two transmembrane domains and a zinc-
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24 binding motif, probably involved in the poly-ubiquitination of the peroxisomal import
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26 receptor *PEX5* (Platta et al., 2009). The *PEX2* gene contains four exons, but the entire coding
27
28 region is included in exon 4. One mutation in the *PEX2* gene (c.355C>T) was found to be
29
30 associated with temperature sensitivity: cells cultured at 30°C show partial catalase import
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32 (Imamura et al., 1998b). The patient fibroblasts homozygous for the c.669G>A mutation
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34 displayed a mosaic peroxisomal pattern, characterized by import competency of catalase in
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36 30% of the cells (Gootjes et al., 2003; Gootjes et al., 2004a). In 3 different cell lines, sequence
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38 analysis of exon 4 identified only one heterozygous mutation (table 1). We did not analyze the
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40 remaining exons, promoter sequence or *PEX2* cDNA of these cell lines. In this study, six
41
42 novel mutations have been identified, making a total of 17 different mutations currently
43
44 identified in the *PEX2* gene (table 3). Two novel missense mutation are identified which are
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46 not listed in the SNP database, and both affect (well) conserved amino acids. In addition,
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48 Polyphen predicts these amino acid substitutions as probably damaging.
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2 PEX3 (MIM603164) is an integral membrane protein with two putative membrane-
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4 spanning domains. The exact function of PEX3 is unclear, but it has been assumed that PEX3
5
6 plays a role in insertion of peroxisomal membrane proteins into the peroxisomal membrane
7
8 based on the absence of peroxisomal ghosts in cell lines with a defect in PEX3 (Ghaedi et al.,
9
10 2000; Muntau et al., 2000). In total, six different mutations in *PEX3* were identified of which
11
12 four have been reported previously (table 4).

13
14 PEX5 is a protein with seven di-aromatic pentapeptide repeats (WxxxF/Y) essential
15
16 for docking to the peroxisomal membrane and for binding to either PEX13 (MIM601789) or
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18 PEX14 (MIM601791), (Saidowsky et al., 2001; Weller et al., 2003), and with seven
19
20 tetrapeptide repeats (TPRs) to mediate the interaction with the PTS1 sequence. In humans,
21
22 two functional protein variants of PEX5 are produced as a result of alternative splicing of the
23
24 *PEX5* mRNA. The longest variant, PEX5L, contains an additional 111 bp encoding 37 amino
25
26 acids, due to alternative splicing of exon 7 (Dodt et al., 2001). The shorter protein, PEX5S,
27
28 has been reported to be exclusively involved in peroxisomal PTS1 protein import, whereas
29
30 PEX5L mediates both PTS1 and PTS2 protein import. We recently published [and discussed](#)
31
32 [the mutations we identified](#) in the *PEX5* gene [elsewhere](#) (Ebberink et al., 2009b). These
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34 mutations combined with previously reported mutations are listed in table 5.

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36 The *PEX6* gene consists of 17 exons and mutations in the *PEX6* gene were found to be
37
38 scattered throughout the whole gene. PEX6 is also a member of the AAA ATPase family and
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40 interacts with PEX1. PEX6 is required for import of peroxisomal matrix proteins and/or
41
42 vesicle fusion. At present, 77 different mutations in *PEX6* gene have been identified, a
43
44 complete overview of which has been reported very recently and thus will not be shown
45
46 here (Ebberink et al., 2009a).

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48 PEX10 (MIM602859) is an integral membrane protein and contains two
49
50 transmembrane domains and a C-terminal zinc-binding motif, [similar as](#) PEX2 and PEX12.

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2 Two mRNA splice forms of *PEX10* have been identified, resulting from the use of a different
3 splice acceptor site at the 3'end of intron 3. The longer form accounts for 10% of the *PEX10*
4 mRNA in the cell and appears to be slightly less functional (Warren et al., 2000). Combined
5
6 with previously reported *PEX10* mutations, 25 different mutations have been identified in the
7
8 *PEX10* gene of which nine have not been reported previously (table 6). The six novel
9
10 missense mutations all affect well conserved amino acids and Polyphen predicts these amino
11
12 acid substitutions as probably damaging. Sequence analysis of the exons plus flanking
13
14 intronic sequences of the *PEX10* gene identified in one patient only one heterozygous
15
16 mutation. We did not analyze the *PEX10* cDNA in this cell line.

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20 Mutations in the *PEX12* gene are the third common cause of a ZSS disorder: 9% of
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22 our patients group has mutations in *PEX12*. *PEX12* is one of the RING (abbreviation for
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24 really interesting new gene) finger proteins; it contains two transmembrane domains and a C-
25
26 terminal zinc-binding motif. All 34 identified *PEX12* mutations are listed in table 7, including
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28 8 that have not been reported before. The pS320F mutation (c.959C>T) is the most common
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30 *PEX12* mutation and is located in the zinc-binding domain. Patients homozygous for this
31
32 mutation present with an atypical biochemical phenotype in skin fibroblasts: the peroxisomal
33
34 parameters are mostly normal to slightly abnormal. The mutation causes a temperature-
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36 sensitive phenotype: when cultured at 30°C, all cells contain catalase import competent
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38 peroxisomes, when cultured at 37°C only, 70% of the cells contain catalase-positive
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40 peroxisomes and when cultured at 40°C none of the cells contain catalase-positive
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42 peroxisomes (Gootjes et al., 2004c). We identified two novel missense mutations both
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44 affecting well conserved amino acids and Polyphen predicts these amino acid substitutions as
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46 probably damaging.

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48 *PEX13* is a peroxisomal integral membrane protein with two putative membrane
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50 domains. The C-terminus contains an SH3 domain, which binds to pentapeptide repeats 2-4 of
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1
2 PEX5. The N-terminal part of yeast PEX13 interacts with PEX7. Table 8 lists ten different
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4 *PEX13* mutations of which six have been reported before. The I326T mutation causes a
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6 temperature-sensitive biochemical phenotype in fibroblasts: peroxisomes still import residual
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8 levels of PTS1 and PTS2 proteins at 37°C but not at elevated temperature (Liu et al., 1999;
9
10 Shimozawa et al., 1999a). One novel missense mutation was identified which affects a well
11
12 conserved amino acid and is probably damaging according to Polyphen.
13

14 The *PEX14* gene encodes a peroxisomal membrane protein that was initially identified
15
16 as a peroxisomal docking factor for the PTS1 receptor PEX5 via binding to the amino-
17
18 terminal WxxxF/Y motifs of PEX5. More recently, it was proposed that PEX14 is also the
19
20 site from which PEX5 leaves the peroxisomal compartment (Azevedo and Schliebs, 2006). A
21
22 mutation in *PEX14* is the least common cause of ZZS disorder. Three different mutations
23
24 have now been identified in the *PEX14* gene of which 1 has not been reported previously
25
26 (table 9).
27

28 The *PEX16* (MIM603360) gene encodes an integral peroxisomal membrane protein
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30 involved in peroxisomal membrane assembly. Two different groups of PEX16-defective
31
32 patients have been reported; patients with a severe clinical presentation of which the
33
34 fibroblasts displayed a defect in import of peroxisomal matrix and membrane proteins,
35
36 resulting in a total absence of peroxisomal remnants (Honsho et al., 1998; Shimozawa et al.,
37
38 2002), and very recently, several patients with a relatively mild clinical phenotype of which
39
40 the fibroblasts showed enlarged, import-competent peroxisomes (Ebberink et al., 2010). In
41
42 total eight different mutations have been identified in the *PEX16* gene of which one has not
43
44 been reported previously (table 10).
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46 The *PEX19* (MIM600279) gene encodes a mainly cytosolic protein that binds many
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48 peroxisomal membrane proteins, suggesting that PEX19 functions as a peroxisomal receptor
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50 for peroxisomal membrane proteins (Sacksteder et al., 2000). The patients with a defect in
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2 PEX19 presented with a severe ZS phenotype and the fibroblasts displayed the absence of
3 peroxisomal ghosts (Matsuzono et al., 1999). Table 11 lists three different *PEX19* mutations
4 of which two have not been reported before.
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7
8 PEX26 (MIM608666) is a membrane protein with one putative membrane-spanning
9 domain. Human PEX26 is the orthologue of yeast PEX15. Some of the reported mutations
10 were found to lead to temperature-sensitive biochemical phenotype (Matsumoto et al., 2003b;
11 Matsumoto et al., 2003a). Combined with previously reported *PEX26* mutations, 22 different
12 mutations have now been identified in the *PEX26* gene of which three have not been reported
13 previously (table 12).
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20 21 22 *Conclusions*

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24 In summary, we developed an efficient genetic complementation assay, which allows rapid
25 identification of the defective *PEX* gene in fibroblast cell lines of ZSS patients. This assay has
26 been included in our diagnostic work up of patients diagnosed with a ZSS disorder, followed
27 by sequence analysis of the defective *PEX* gene. After analysis of 613 independent cell lines,
28 we did not detect cell lines with a novel defective *PEX* gene suggesting that only mutations in
29 one of the currently known *PEX* genes leads to complete peroxisome deficiency and a
30 Zellweger Syndrome-like presentation.
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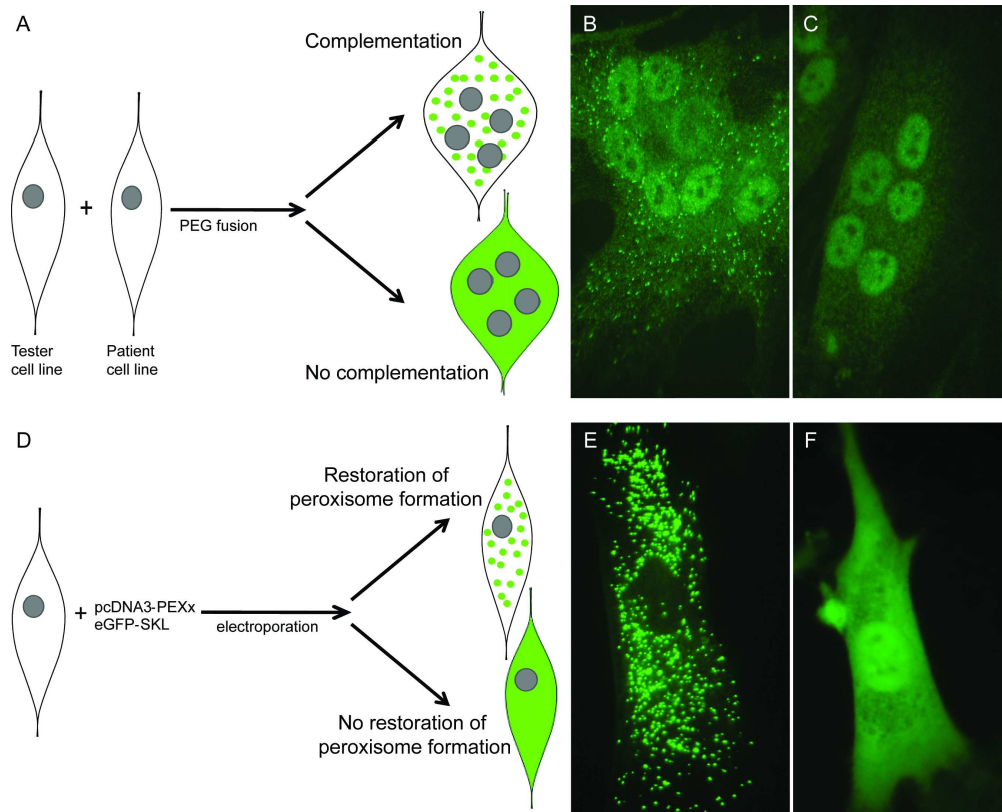
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4 **Figure 1. Complementation testing by PEG fusion and by *PEX* cDNA transfection.**

5 Principle of the PEG fusion method. (A), a tester cell line with a known *PEX* gene defect and
6 a patient cell line are fused by PEG treatment resulting in multi-nucleated cells. Cells are
7 examined by catalase immunofluorescence microscopy to determine complementation (B) or
8 non complementation (C). Principle of the *PEX* cDNA transfection assay. The pcDNA3-*PEX*
9 expression vectors are co-transfected separately but together with eGFP-SKL into patient
10 cells (D). Cells are examined by direct fluorescent microscopy to determine complementation
11 (E) or non complementation (F).
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For Peer Review



Complementation testing by PEG fusion and by PEX cDNA transfection.
228x185mm (300 x 300 DPI)

Table 1. Frequency distribution of *PEX* gene defects in ZSS.

Classification of the 613 different patient cell lines was done by either genetic complementation (cDNA transfection of PEG fusion) or by sequence analysis.

Deleted: 0

PEX gene	Genetic complementation (Number of cell lines)	Frequency (%)	Number of cell lines sequenced <u>and mutations</u>			
			Total	Homozygous ¹	2x Heterozygous	1x Heterozygous
PEX1	358	58	180	74	68 ²	38 ³
PEX2	23	4	22	17	2	3 ^{4,5}
PEX3	3	<1	3	3		
PEX5	13	2	13	13		
PEX6	97	16	77	45	32	
PEX10	19	3	18	10	7	1 ⁵
PEX12	54	9	43	29	14	
PEX13	10	1	7	7		
PEX14	3	<1	2	1		1 ⁵
PEX16	8	1	8	8		
PEX19	4	<1	3	3		
PEX26	21	3	17	15	2	
Total	613		393			

¹These mutations appear homozygous, but a genomic deletion of one allele cannot be excluded.

²This number of cell lines includes 2 cell lines with 3 heterozygous mutations.

³Mutation analysis of the *PEX1* gene by RFLP analysis identified the p.G843D mutation heterozygous in 38 patients; the *PEX1* gene in these patients was not further screened by sequence analysis.

⁴Only coding exon 4 was sequenced.

⁵We did not analyze the corresponding cDNA in these cell lines

Table 2. Mutations in the *PEX1* gene.

Exon	Mutations Nucleotide	Amino acid	Number of patients identified in this study		Reference
			Homozygous	Heterozygous	
1	c.2T>C	unknown	3		this study
1	c.3G>A	unknown		1	this study
1	c.5G>A	p.W2X		1	this study
1	c.56_57delTG	p.V19GfsX48		1	this study
2	c.130-2A>T	-		1	this study
i2	c.273+1G>A	-		1	this study
3	c.274G>C	p.V92L			(Rosewich et al., 2005)
3	c.343_344insCT	p.D115AfsX18	1		this study
4	c.434_448delinsGCAA	p.V145_Q150delinsGfsX24		1	(Walter et al., 2001)
5	c.547C>T	p.R183X		1	(Yik et al., 2009)
5	c.569C>A	p.S190X		4	this study
5	c.616C>T	p.Q206X		1	this study
5	c.781C>T	p.Q261X			(Tamura et al., 2001)
5	c.782-783delAA	p.Q261RfsX8	3		(Yik et al., 2009)
5	c.788_789delCA	p.T263fsX5		1	(Walter et al., 2001)
5	c.904delG	p.A302QfsX23			(Maxwell et al., 2005; Maxwell et al., 2002)
5	c.911_912delCT	p.S304fsX3		1	(Rosewich et al., 2005)
5	c.1007T>C	p.V336A ¹		1	this study
5	c.1108_1109insA	p.I370NfsX2	1		(Maxwell et al., 2005)
5	c.1193delT	p.I398TfsX7		1	this study
i5	c.1239+1G>T	-		1	(Yik et al., 2009)
6	c.1340T>C	p.L447S		1	this study
7	c.1370insT	p.S458KfsX2		1	this study
7	c.1411C>T	p.Q471X		1	this study

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4	7	c.1414C>T	p.Q472X	1	this study
5	8	c.1501_1502delCT	p.L501X	1	this study
6	8	c.1509_1510dupTA	p.V504X	1	this study
7	8	c.1579A>G	p.T526A ¹	1	this study
8	9	c.1663T>C	p.S555P	1	this study
9	i9	c.1670+5G>T	-	1	(Walter et al., 2001)
10	10	c.1706A>C	p.H569P	1	this study
11	10	c.1713_1716delTCAC	p.H571fsX10	1	this study
12	10	c.1716_1717delCA	p.H572QfsX19	2	(Yik et al., 2009)
13	10	c.1777G>A	p.G593R	1	(Walter et al., 2001)
14	11	c.1842delA	p.E615KfsX30	1	this study
15	11	c.1865_1866insCAGTGTGGA	-		(Preuss et al., 2002)
16	11	c.1886_1887delGT	p.C629X	1	this study
17	11	c.1891delG	p.A631LfsX14	1	this study
18	11	c.1897C>T	p.R633X		(Tamura et al., 2001)
19	12	r.1900_2070del	p.G634_H690del		(Tamura et al., 1998)
20	12	c.1957T>A	p.W653R	1	this study
21	12	c.1960_1961dupCAGTGTGGA	p.T651_W653dup	1	(Gartner et al., 1999; Portsteffen et al., 1997; Preuss et al., 2002; Reuber et al., 1997)
22	12	c.1976T>A	p.V659D	1	this study
23	12	c.1991T>C	p.L664P		(Tamura et al., 2001)
24	12	c.2008C>A	p.L670M	1	(Walter et al., 2001)
25	12	c.2034_2035delCA	p.H678QfsX15	1	(Steinberg et al., 2004)
26	12	c.2071+1G>T	-	5	(Walter et al., 2001)
27	13	c.2083_2085delATG	p.M695del		(Rosewich et al., 2005)
28	13	c.2085-2089delGATAA	p.M695lfsX45		(Steinberg et al., 2004)
29	13	c.2097_2098insT	p.I700fsX41	16	(Collins and Gould, 1999; Maxwell et al., 2005; Preuss et al., 2002; Steinberg et al., 2004; Walter
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					et al., 2001)	
	13	c.2224C>T	p.Q742X	2	this study	
	13	c.2227_2416del	p.E743NfsX2fs		(Preuss et al., 2002)	
	14	c.2364G>A	Deletion exon 14	1	this study	Deleted: p.V788V
	14	c.2368C>T	p.R790X	1	(Maxwell et al., 2002)	Deleted: 2
	14	c.2383C>T	p.R795X	1	(Collins and Gould, 1999)	
	14	c.2387T>C	p.L796P	1	(Rosewich et al., 2005)	
	14	c.2391_2392delTC	p.R798SfsX35		(Maxwell et al., 2005)	
	14	c.2392C>G	p.R798G	1	(Maxwell et al., 2002)	
	15	c.2528G>A	p.G843D	42	62 (Collins and Gould, 1999; Maxwell et al., 2002; Preuss et al., 2002; Steinberg et al., 2004; Walter et al., 2001)	Deleted: 4 Deleted: 9
	15	c.2537_2545delATGAAGTTAinsTCATGGT	p.H846_R849delinsLfsX53		(Steinberg et al., 2004)	
	15	14bpinsex15	-		(Reuber et al., 1997)	
	16	c.2614C>T	p.R872X	2	1 (Preuss et al., 2002)	Deleted: 1
	16	c.2633_2635 delTGT	p.L879del	1	1 this study	
	16	c.2636T>C	p.L879S	1	3 this study	Deleted: 2
	16	c.2654C>G	p.T885R	1	1 this study	
	17	c.2730delA	p.I910fsX50	1	4 (Walter et al., 2001)	
	17	c.2760delA	p.A921LfsX40	1	this study	
	18	c.2814_2818delCTTTG	p.F938LfsX2		(Preuss et al., 2002)	
	18	c.2845C>T	p.R949W		1 this study	
	18	c.2846G>A	p.R949Q		2 (Walter et al., 2001)	
	18	c.2916delA	p.G973AfsX16	1	4 (Maxwell et al., 2002; Preuss et al., 2002; Steinberg et al., 2004)	
	18	c.2926+1G>A	-		1 (Steinberg et al., 2004)	
	18	c.2926+2_2926+3insT	-		(Collins and Gould, 1999)	
	18	c.2926+2T>C	-		(Walter et al., 2001)	

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	c.2927-15T_3208-			
19	342AdelinsATAGTATAGA & c.3849+779_3849+2910	del ex19/20	2	this study
19	c.2966T>C	p.I989T		(Maxwell et al., 2005)
19	c.2992C>T	p.R998X		(Maxwell et al., 2002)
19	c.2993G>A	p.R998Q		(Maxwell et al., 2005)
19	c.3022_3024delCCT	p.P1008del		(Steinberg et al., 2004)
	20 c.3038G>A	p.R1013H		(Rosewich et al., 2005)
20	c.3180_3181insT	p.G1061WfsX16		(Collins and Gould, 1999)
20	c.3207+1G>C	-		(Collins and Gould, 1999)
	21 c.3287C>G	p.S1096X		(Rosewich et al., 2005)
	21 c.3378C>G	p.Y1126X		(Preuss et al., 2002)
23	c.3692-3695delAGTC	p.Q1231HfsX3	1	(Yik et al., 2009; Rosewich et al., 2005)
24	c.3850T>C	p.X1284QnextX29	1	(Walter et al., 2001)

Reference sequence of *PEX1*: GenBank accession number NM_000466.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

¹[Possibly this mutation is a rare sequence variation, for details see text.](#)

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13	c.2088A>G	p.I696M	1

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Table 3. Mutations in the *PEX2* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
4	c.115C>T	p.Q39X			(Krause et al., 2006)
4	c.163 G>A	p.E55K			(Imamura et al., 1998b; Steinberg et al., 2004)
4	c.273delT	p.N92TfsX2			(Steinberg et al., 2004)
4	c.279_283delGAGAT	p.R94SfsX5	2	1	(Gootjes et al., 2004a)
4	c.286C>T	p.Q96X	1		this study
4	c.325insT	p.C109LfsX14		1	this study
4	c.339_345delCAGGTGG	p.R114X	1	1	this study
4	c.355C>T	p.R119X	4	1	(Imamura et al., 1998b; Shimozawa et al., 1993; Steinberg et al., 2004)
4	c.373C>T	p.R125X			(Imamura et al., 1998b; Shimozawa et al., 1998; Steinberg et al., 2004)
4	c.550delC	p.R184VfsX8	3		(Shimozawa et al., 2000b)
4	c.642delG	p.K215SfsX2	1		(Shimozawa et al., 2000b)
4	c.669G>A	p.W223X	2		(Gootjes et al., 2004a)
4	c.739T>C	p.C247R	1		(Gootjes et al., 2004a)
4	c.782A>G	p.H261R		1	this study
4	c.791G>A	p.C264Y	1		this study
4	c.834_838delTACTT	p.F278LfsX3		1	(Krause et al., 2006)
4	c.866insA	p.S289fsX36		1	this study

Reference sequence of *PEX2*: GenBank accession number NM_000318.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

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Table 4. Mutations in the *PEX3* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
2	c.157C>T	p.R53X			(South et al., 2000)
4	c.328_331delATAA	p.I110VfsX23	1		this study
5	r.334_393del	p.F112_V131del	1		this study
7	c.543_544insT	p.V182CfsX2			(Muntau et al., 2000; Shimozawa et al., 2000a; South et al., 2000)
10	c.856c>t	p.R286X	1		(Dursun et al., 2009)
11	c.942-8T>G	p.S314RfsX3			(Ghaedi et al., 2000; Muntau et al., 2000)

Reference sequence of *PEX3*: GenBank accession number NM_003630.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

Table 5. Mutations in the *PEX5* gene.

Exon	Nucleotide	Mutations Amino acid	Number of patients identified in this study		Reference
			Homozygous	Heterozygous	
5	c.548_552-55delins238inv	p.D150GfsX33	1		(Ebberink et al., 2009b)
7	c.826C>T	p.R276X	1		(Ebberink et al., 2009b)
10	c.1090C>T	p.Q364X	1		(Ebberink et al., 2009b)
11	[c.1244A>G]+[c.548-549dupATCG; c.604G>C]	p.N415S	1		(Ebberink et al., 2009b)
i12	c.1394+5G>A	p.R394S, r.395_465del	1		(Ebberink et al., 2009b)
12	c.1258C>T	p.R420X	2		(Ebberink et al., 2009b)
12	c.1279C>T	p.R427X			(Dodt et al., 1995)
i14	c.1561-2A>C	r.1561_1718del and r.1561_1584del	1		(Ebberink et al., 2009b)
14	c.1578T>G	p.N526K	2		(Dodt et al., 1995; Ebberink et al., 2009b)
14	c.1669C>T	p.R557W	1		(Ebberink et al., 2009b)
15	c.1799C>G	p.S600W	1		(Ebberink et al., 2009b; Shimozawa et al., 1999b)

Reference sequence of *PEX5L*: GenBank accession number X84899. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

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Table 6. Mutations in the *PEX10* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
1	c.2T>C	p.M1T		1	(Regal et al., 2010)
1	c.3G>A	p.M1I		1	this study
1	c.4delG	p.A2PfsX9			(Steinberg et al., 2004)
1	c.13_28del, ins20bp	p.A5PfsX46			(Warren et al., 2000)
3	c.203C>A	p.T68N		1	this study
3	c.208G>C	p.G70R		1	this study
3	c.211G>A	p.E71K	1		this study
3	c.233A>G	p.Q78R	1		this study
3	c.337delC	p.L113WfsX40			(Steinberg et al., 2009)
3	c.352C>T	p.Q118X	1		this study
3	c.373C>T	p.R125X			(Warren et al., 1998)
i3	c.600+1G>A	-	1		(Warren et al., 1998)
4	c.646_647insA	p.S216KfsX123	1		this study
4	c.697delG	p.A213PfsX131	1		this study
4	c.704_705insA	p.L236AfsX102	2	2	(Steinberg et al., 2004; Warren et al., 2000)
4	c.730C>T	p.R244X		1	(Krause et al., 2006; Steinberg et al., 2004)
5	c.814_815delCT	p.L272VfsX65	1	1	(Okumoto et al., 1998a)
5	c.830T>C	p.L277P			(Steinberg et al., 2009)
5	c.835G>T	p.E279X		1	(Steinberg et al., 2004)
5	c.868C>G	p.H290D			(Steinberg et al., 2004)
5	c.870C>G	p.H290Q			(Warren et al., 1998)
5	c.881G>A	p.W294X			(Krause et al., 2006)
5	c.892G>T	p.C301F			(Steinberg et al., 2004)
6	c.919T>C	p.C307R	1		this study

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6 c.932G>A p.R311Q 1 (Regal et al., 2010)
Reference sequence of *PEX10*: GenBank accession number NM_002617.3. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

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Table 7. Mutations in the *PEX12* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
1	c.16dupG	p.A6GfsX11		1	this study
1	c.26_27delCA	p.T9SfsX7			(Chang and Gould, 1998)
1	c.102A>T	p.R34S	1		(Zeharia et al., 2007)
1	c.126+1G>T	-	3	5	(Chang and Gould, 1998)
2	c.202_204delCTT	p.L68del			(Chang and Gould, 1998)
2	c.220T>G	p.Y74D		1	this study
2	c.260_261insAA	p.Y87X			(Steinberg et al., 2004)
2	c.268_271delAAGA	p.K90EfsX3			(Chang and Gould, 1998)
2	c.273A>T	p.R91S	1		(Gootjes et al., 2004c)
2	c.308_309insT	p.L103FfsX3	1		(Gootjes et al., 2004c)
2	c.378_380delTTC	p.L126del		1	this study
2	c.445_454delTCTTCCCGCT	p.S149GfsX15		1	this study
2	c.460C>T	p.R154X		1	this study
2	c.478_479delCA	p.Y156X		1	this study
2	c.531_533delACA	p.Q178del			(Yik et al., 2009)
2	c.533_535delAAC	p.Q178del	1	1	(Steinberg et al., 2004)
2	c.538C>T	p.R180X		2	(Chang et al., 1999; Okumoto et al., 1998b)
2	c.541_542insT	p.Y181LfsX37			(Steinberg et al., 2004)
2	c.604C>T	p.R202X	3		(Gootjes et al., 2004c)
2	c.625C>T	p.Q209X		2	(Gootjes et al., 2004c)
i2	c.681-2A>C	-			(Steinberg et al., 2004)
3	c.684_687delTAGT	p.S222RfsX3			(Chang and Gould, 1998)
3	c.691A>T	p.K231X			(Okumoto and Fujiki, 1997)
3	c.733_734dupGCCT	p.L245CfsX19		1	(Chang et al., 1999; Steinberg et al., 2004)

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3	c.744_745insT	p.T249YfsX14		1	(Chang and Gould, 1998)
3	c.775C>T	p.Q259X	1		this study
3	c.875_876delCT	p.S292X	1		(Okumoto et al., 1998b)
3	c.887_888delTC	p.L296fs3007X	1	1	(Chang et al., 1999; Gootjes et al., 2004c; Steinberg et al., 2004)
3	c.887delT	p.L296PfsX5			(Steinberg et al., 2004)
3	c.888_889delCT	p.L297TfsX12	1	3	(Chang and Gould, 1998; Steinberg et al., 2004)
3	c.920G>A	p.C307Y		1	this study
3	c.949C>T	p.L317F		1	(Gootjes et al., 2004d)
3	c.959C>T	p.S320F	1 3	2	(Steinberg et al., 2004)
3	c.1047_1049delACA	p.Q349del	2		(Steinberg et al., 2004)

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Reference sequence of *PEX12*: GenBank accession number NM_000286.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

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Table 8. Mutations in the *PEX13* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
i1	c.92+2T>G	-	1		this study
2	c.107_120del	p.G36DfsX61			(Al-Dirbashi et al., 2009)
2	c.439_441delATG	p.M147del	1		this study
2	c.676C>T	p.R226X	2		this study
2	c.702G>A	p.W234X	1		(Shimozawa et al., 1999a)
4	c.937T>G	p.W313G			(Krause et al., 2006)
4	c.970G>T	p.G324X			(Shimozawa et al., 1999a)
4	c.977T>C	p.I326T	1		(Liu et al., 1999; Shimozawa et al., 1999a)
4	c.980c>g	p.P327R	1		this study
1-4	c.1_1212 ¹	-			(Al-Dirbashi et al., 2009)

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Reference sequence of *PEX13*: GenBank accession number NM_0002618.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

¹Deletion of 147,308 bp that included the *PEX13* gene and 70,094 bp upstream and 45,692 bp downstream of the *PEX13* the gene.

Table 9. Mutations in the *PEX14* gene.

Exon	Nucleotide	Mutations Amino acid	Number of patients identified in this study		Reference
			Homozygous	Heterozygous	
3	c.85-?_170+?del	p.[Ile29_Lys56del;Gly57GlyfsX2]	<u>1</u>		(Huybrechts et al., 2008)
7	c.553C>T	p.Gln185Ter			(Shimozawa et al., 2004)
7	c.585+1G>T	r.585+1_37ins		<u>1</u>	this study

Reference sequence of *PEX14*: GenBank accession number NM_004565.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

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Table 10. Mutations in the *PEX16* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
i5	c.460+2 insG	-	1		this study
6	c.526C>T	p.R176X			(Honsho et al., 1998; South and Gould, 1999)
8	c.753_755delTGT	p.V252del	1		Ebberink 2010
9	c.865C>A	p.P289T	1		Ebberink 2010
i11	c.952+118_1011+80		1		Ebberink 2010
i10	c.952+2T>C	R298fsX38	2		(Shimozawa et al., 2002)
11	c.984delG	p.I330SfsX27	1		Ebberink 2010
11	c.992A>G	p.Y331C	1		Ebberink 2010

Reference sequence of *PEX16*: GenBank accession number NM_000481.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

Table 11. Mutations in the *PEX19* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
3	c.320delA	p.K320fsX13	1		this study
6	c.739c>t	p.Q257X	1		this study
6	c.763_764insA	p.M255NfsX24	↓		(Matsuzono et al., 1999)

Reference sequence of *PEX19*: GenBank accession number NM_0002857.2. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

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Table 12. Mutations in the *PEX26* gene.

Exon	Mutations		Number of patients identified in this study		Reference
	Nucleotide	Amino acid	Homozygous	Heterozygous	
2	c.2T>C	p.M1T			(Matsumoto et al., 2003b; Weller et al., 2005)
2	c.34_35insC	p.L12PfsX103	2		(Matsumoto et al., 2003b)
2	c.37_38delAG	p.R13GfsX164			(Steinberg et al., 2004)
2	c.73_79delGTGCGCG	p.V25RfsX55	1		this study
2	c.131T>C	p.L44P		1	(Weller et al., 2005)
2	c.134T>C	p.L45P			(Matsumoto et al., 2003b; Weller et al., 2005)
2	c.185G>A	p.W62X	1		this study
2	c.192_216del	p.S64RfsX10			(Steinberg et al., 2004)
i2	c.230+1G>T	p.T77fsX139			(Weller et al., 2005)
3	c.254_255insT	p.L85LfsX93			(Matsumoto et al., 2003b; Weller et al., 2005)
3	c.265G>A	p.G89R			(Matsumoto et al., 2003b)
3	c.292C>T	p.R98W	10	2	(Matsumoto et al., 2003b; Steinberg et al., 2004; Weller et al., 2005)
3	c.296G>A	p.W99X			(Steinberg et al., 2004)
3	c.315g>a	p.W105X	1		this study
3	c.350C>T	p.P117L			(Weller et al., 2005)
3	c.353C>G	p.P118R			(Steinberg et al., 2004)
i3	c.371+2T>C	-			(Steinberg et al., 2004)
4	c.426_548dup122bpinsT	p.A143_V182dup+G183V			(Weller et al., 2005)
4	c.574C>T	p.R192X		1	(Steinberg et al., 2004)
i4	c.667+2T>C	-			(Steinberg et al., 2004)
5	r.668_814	p.G223_P271del			(Matsumoto et al., 2003b)
6	c.862delC	p.R288AfsX366			(Weller et al., 2005)

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Reference sequence of *PEX26*: GenBank accession number NM_017929.4. Nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

Supplemental Table S1. Primer sets used for *PEXI* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Exons	Regions of cDNA
A	[-21M13]-ACTACAGGCTTACGGCAG [M13-Rev]-GACCTGGGATAAGACGAGC	1	
B	[-21M13]-CAGCATTGGGTGTTGGAAATG [M13-Rev]-ATTAGGTGACATCTCACCTCC	2	
C	[-21M13]-CCATCCAAAACATACACACAC [M13-Rev]-AAGCTAAAGACATTGATATTGTG	3	
D	[-21M13]-TTAACTAGATATGGAGTGGAC TACTTTGGACTCAATGCTATAG	4	
E	[-21M13]-TATAGCATTGAGTCCAAAGTAG [M13-Rev]-CTGGTCCCATGGAAATACATG	5A	
F	[-21M13]-CAGAGTATGCAAATCTCAACC [M13-Rev]-ATTTGGGGATGTTTAAGCCAC	5B	
G	[-21M13]-ATGGACTGAGACAACATGAG [M13-Rev]-CTAAAGCAACTAGAAATCTTAC	6	
H	[-21M13]-GATTTGTTTGGTCAGGCTTAC [M13-Rev]-CAGGTATGACAGGTTGCAAG	7	
I	[-21M13]-CTGTTTCAGTACTAACTCTGC [M13-Rev]-CTTCACAATGCAAGGTGTTAC	8	
J	[-21M13]-CCCTGGATGGGTACATTGTT [M13-Rev]-CTAGGCGTGAAGATAGAGAC	9	
K	[-21M13]-TATCCTGCTTCAGTCTTTTATC [M13-Rev]-TTTCCATATGTCGTATAAACCC	10	
L	[-21M13]-GTGGTTGCCACAATGACAGG [M13-Rev]-AGATTGACAGCATTATGTATAAC	11	
M	[-21M13]-CCTGAATTTTCCCAGCACTG [M13-Rev]-GACTAACCATGAAGTTGCAGA	12	
N	[-21M13]-CCTCACTAGACATTGCTCTG [M13-Rev]-CCTCTAGCACAATATGCACC	13	
O	[-21M13]-GCCCTTTTACTACCCATTCC [M13-Rev]-TACAGTGGTTCTTCTGGGAG	14 + 15	
P	[-21M13]-TTTTCTACTGTTGTACTTGCTG [M13-Rev]-TGAATGCACTAAATGCCAGTG	16	
Q	[-21M13]-GGCAGAAGCATCAAGCATAG [M13-Rev]-AGAGAATCCACGTCCTCTAG	17	
R	[-21M13]-GCCAACTATGAAGCCTGA TTC [M13-Rev]-CTGGCAGAAGTAAAGCTCAC	18 + 19	
S	[-21M13]-GCTGTTGGTACATTTCTCAAC [M13-Rev]-TCCAACATGGAACATTCAACT	20	
T	[-21M13]-TGAGTCTTAGCTTCATTACAGA [M13-Rev]-TAGGAGAGAAATCACTGCAAC	21	
U	[-21M13]-TTTTTCTCTCCCCCTCTTCC [M13-Rev]-TTGTAATAGTAGCTGTAATTCC	22 + 23	
V	[-21M13]-TCCTTTGCGGCGCTAGGGTG [M13-Rev]-TTGGCTCGGCGTGCTTTTGG		-48_554
W	[-21M13]-CCAGCTGCCTCTTATGGAAG [M13-Rev]-AGCACACAGGCCTTCTCATC		484_1151
X	[-21M13]-GATGTCAGAGCCACTAGATC [M13-Rev]-ACTCCTCCCAAAGAGCTCAGC		1080_1676
Y	[-21M13]-AAGTCCTTCTAGATCCTATGG [M13-Rev]-GCAGGTCAAGATCGGTGAAC		1586_2308
Z	[-21M13]-TCAGCCTCCTAATCAGGAAC		2211_2866

AA	[M13-Rev]-CTCCTGTATTATCATGACCC [-21M13]-GCAAGTGAACAAGCTGTTTCG	2749_3388
AB	[M13-Rev]-AGTAGAGCCGGTACATATTG [-21M13]-TCAGTCCCTTGTCTTTAG [M13-Rev]-CAACATATGGAAAAGCCATC	3315_3910
RFLP	CCTGGGTTGGGACAAGATCG [M13-Rev]-ATCGATACAGTGGTCTTCTGGGAG	

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S2. Primer sets used for *PEX2* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Exons
A	[M13-Rev]-TCAGAGACAGAGTTCTTCCG [-21M13]-CAGGAAGCCAATAAACAGGG	1
B	[M13-Rev]-ACTGAAGGCTCAGATGGTTG [-21M13]-TGGTCTTCACCATCACAGTC	2
C	[M13-Rev]-TTAGAACAACACTGGCAGTGTGG [-21M13]-ATGCTTCTCACCATAAATGCC	3
D	[M13-Rev]-AAACGCTCATCGCCTATGTG [-21M13]-GTTGCAAACCTTCCCTCTG	4A
E	[M13-Rev]-TGGGAAAGTCAAGCAGTGTG [-21M13]-TCCTCAAAGGAAGCAATTTAG	4B

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S3. Primer sets used for *PEX3* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Regions of cDNA
A	[-21M13]- CTCACCCCTAGTCAGCCCAC [M13-Rev]- CCATCTCCAAGTAGGTGCTG	-17_505
B	[-21M13]- CTGGATAATGCAGCAGTTGG [M13-Rev]- TGTTGCAGGTCCTGTTCAAGTA	424_903
C	[-21M13]- ATGCCAGATGAAGAACTCC [M13-Rev]- TGCCACAAAACTGGTCTG	715_1123+186

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S4. Primer sets used for *PEX5* mutation analysis

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-ACGGGCAGAGTTGTGGATG [M13-Rev]-ATTGAA ATACGGGTGAACTAAG	1 + 2
B	[-21M13]-AGCCTATGGGTTTCATTTATC [M13-Rev]-AGAATTCTGTCCCATAGAAGC	3 + 4
C	[-21M13]-TCAGTTGAATATGGGCATCTC [M13-Rev]-TGTCCTACTCCTTTTAC	5
D	[-21M13]-ACAGGAACTGTCATTGTGTCATG [M13-Rev]-CAGGAACGAAGAGACCTAAG	6

E	[-21M13]-TGGAAGTCCTTTCCCAAGTG [M13-Rev]-TCCAGGTCCACTATGAAATAC	7 + 8
F	[-21M13]-TCAAATTCAAGAAGTCTGCTGCC [M13-Rev]-GAAGGAAGTTCTGGAACCTG	9
G	[-21M13]-CTGCCTGCTGGTTGTCATC [M13-Rev]-AAGACAAGGATCCAGGTCTG	10 + 11
H	[-21M13]-AGCTTGGCTTGGATCCAG [M13-Rev]-ACAGGCATGCACCATCAAAC	12 + 13
I	[-21M13]-CCTGGAGTAATGTGCAGAG [M13-Rev]-GTACCGCTTATGGTTCATCAG	14 + 15

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S5. Primer sets used for *PEX10* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-GCTGCGCTGGTTCGGACT [M13-Rev]-GGTGCCCCGCACATCCC	1
B	[-21M13]-GGAGACAGAAGCAGAGAGG [M13-Rev]-CAGCACTGTGACTCACTGG	2
C	[-21M13]-CTCAAGGGCGACAAGATGG [M13-Rev]-CTGGATGTAGAACCCTGTTG	3
D	[-21M13]-GTTTCTGCCGGGCACTCC [M13-Rev]-AACTGGAGGGTGCTCAGAG	4 and 5
E	[-21M13]-CACACCCTGAGCCCTACG [M13-Rev]-AAGCGCAGCCAGGGACAG	6

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S6. Primer sets used for *PEX12* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-TGAGCACCCATCTGATACTC [M13-Rev]-CGCTAGGCTACCAAATAAGC	1
B	[-21M13]-TGTGTCATGGAATGAATTTAC [M13-Rev]-GGGATACGATTTTCGAATTTAC	2
C	[-21M13]-GGAGATAGTACCAGTCTACC [M13-Rev]-TACCATGCTGAAACCAGCTC	3

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S7. Primer sets used for *PEX13* mutation analysis

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-CTACAAATTAGTGGCATCCTC [M13-Rev]-GTGAGACACTAAAGTCCCCAC	1
B	[-21M13]-AGCACCAGGTATATAGGAGATG [M13-Rev]-TTGCTATATATTCCTCTGCCAG	2

C	[-21M13]-GAGGACTTTTCTCTTGGCAG [M13-Rev]-TTTTGTGTGGGGATTAACAG	3
D	[-21M13]-GTTGGACCTCCAAAGTTATTG [M13-Rev]-TATTTCTAGCAGCAAAACACC	4

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S8. Primer sets used for *PEX14* mutation analysis

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-TTAACGGAAACTATGCAAGG [M13-Rev]-AAGGTGACCACAGATGAAGAG	1
B	[-21M13]-GACAAGTGAACCCAGAAACAG [M13-Rev]-CAGAACCATAGGAACAGAAGG	2
C	[-21M13]-TACAGGAAGCCAGTGAAGAC [M13-Rev]-GAGTGGGCAGCTCTAGGAAG	3
D	[-21M13]-AAAGCAGCAATAAACTCAAGG [M13-Rev]-CTCCTGGTTTGCTAAGTCTGG	4
E	[-21M13]-TTCTAACCTCCTCCTCTTC [M13-Rev]-TCCCATTTTACAGACCAGAG	5
F	[-21M13]-TTTATGGTGTGGCTTAGGG [M13-Rev]-CTGTGAGGGGTGACTTGATG	6
G	[-21M13]-CCAGAGTGCAGATAAATTAG [M13-Rev]-GTTGGGAGGTGACATAGAGAG	7
H	[-21M13]-ATCAGATGTCCAAACAGAGC [M13-Rev]-TGGGGAATGAAGAGAGAAGC	8
I	[-21M13]-TCCTGAATGCCGCCACCAAC [M13-Rev]-CAGGTGGACAGAAGTGTGAC	9

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S9. Primer sets used for *PEX16* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-GAAGCAGGAAGGAGGGCG [M13-Rev]-ATTCAGTCATAGCACAAGGTG	1 and 2
B	[-21M13]-TGTGAGATCATGTTGGGGAG [M13-Rev]-CTAAGATGGGAATACTCACAC	3
C	[-21M13]-GTCAGAGAAGCTCCCTCCTAG [M13-Rev]-TACTGTATTCATGCTGGTTGG	4 and 5
D	[-21M13]-CCTGCTTGTAGTTCCTTGAC [M13-Rev]-ATTATAGCAGAAAGCCAGTG	6, 7 and 8
E	[-21M13]-ACATAGGCGGGGTGGCAG [M13-Rev]-CCCGGACAACACACAGTGC	9
F	[-21M13]-GCACGGTGGTCAAGTGAAGG	10 and 11

[M13-Rev]-TATGGCTGCCGAGGCGAG

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S10. Primer sets used for *PEX19* mutation analysis

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-TCCGCACTGGTCAACAATAC [M13-Rev]-TATCATGAAACGCCGTCCTG	1
B	[-21M13]-AGGAGGAAAATTGAGGAAAG [M13-Rev]-CCAATCCAACCATTAAACC	2
C	[-21M13]-TTAAATGGTTGGATTGGTATGC [M13-Rev]-TCCCATCCTTTCTCTCATTG	3
D	[-21M13]-CATAGGGTTTTCCAAGATTG [M13-Rev]-CCTGCCTCAAATAAATAAAG	4 and 5
E	[-21M13]-GTTTTTCTACGTCCCTTCC [M13-Rev]-CATAGCTGGGACTCAGAGAG	6, 7 and 8

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.

Supplemental Table S11. Primer sets used for *PEX26* mutation analysis.

Amplicon	Sequence (5'→3') ¹	Exons
A	[-21M13]-AGCTAGGGCCAGGTATTCC [M13-Rev]-TGATGAAAGCGAGGTGTAAAG	1
B	[-21M13]-AACGTTGAGCAACAGCTGAC [M13-Rev]-GATCTGGAGGGCAATAGTAG	2
C	[-21M13]-GTTAGTGATGGGTGTGTCGT [M13-Rev]-TTCCACTGCCAACTTCAGAG	3
D	[-21M13]-TCTCGATCTCCTGACCTTGT [M13-Rev]-CTAGCAATTCCTCCTTGTGG	4
E	[-21M13]-AGCTGAACACAGACTGCACT [M13-Rev]-CACATCATCTACCGCGACC	5

¹All forward and reverse primers were tagged with a -21M13 (5'-GTAAAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence.