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Allelic Expression Imbalance Promoting a Mutant *PEX6* Allele Causes Zellweger Spectrum Disorder

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Zellweger spectrum disorders (ZSDs) are autosomal-recessive disorders that are caused by defects in peroxisome biogenesis due to bi-allelic mutations in any of 13 different *PEX* genes. Here, we identified seven unrelated individuals affected with an apparent dominant ZSD in whom a heterozygous mutant *PEX6* allele (c.2578C>T [p.Arg860Trp]) was overrepresented due to allelic expression imbalance (AEI). We demonstrated that AEI of *PEX6* is a common phenomenon and is correlated with heterozygosity for a frequent variant in the 3' untranslated region (UTR) of the mutant allele, which disrupts the most distal of two polyadenylation sites. Asymptomatic parents, who were heterozygous for *PEX* c.2578C>T, did not show AEI and were homozygous for the 3' UTR variant. Overexpression models confirmed that the overrepresentation of the pathogenic *PEX6* c.2578T variant compared to wild-type *PEX6* c.2578C results in a peroxisome biogenesis defect and thus constitutes the cause of disease in the affected individuals. AEI promoting the overrepresentation of a mutant allele might also play a role in other autosomal-recessive disorders, in which only one heterozygous pathogenic variant is identified.

Introduction

Peroxisomes are essential organelles involved in multiple metabolic pathways, such as the β -oxidation of branched-chain fatty acids and very long-chain fatty acids (VLCFAs), and the synthesis of plasmalogens and primary bile acids.¹ Defects in genes encoding peroxisomal proteins result in various peroxisomal diseases, including the autosomal-recessive Zellweger spectrum disorders (ZSDs). ZSDs are multisystemic diseases and clinical symptoms can be mild to early lethal, ranging from mild neurosensory deficits to profound neonatal hypotonia and liver dysfunction.² They are caused by bi-allelic mutations in any of 13 different *PEX* genes, which encode proteins involved in peroxisome biogenesis, including the import of peroxisomal proteins.¹ Of these, *PEX1* (~60% [MIM: 602136]) and *PEX6* (~15% [MIM: 601498]) are most commonly defective.³ *PEX1* and *PEX6* both encode AAA+ ATPases, which form hetero-hexameric double-ring complexes composed of equal number of subunits.^{4,5} They are anchored to the cytosolic face of the peroxisomal membrane by the interaction of *PEX6* with

the peroxisomal membrane protein *PEX26*. *PEX1*-*PEX6* complexes facilitate the export of the peroxisomal matrix protein receptor *PEX5* back into the cytosol after *PEX5* has delivered its cargo to the peroxisome (Figure 1A).⁵ Since *PEX5* export is crucial for peroxisomal matrix protein import,⁶ defects in *PEX1* or *PEX6* can prevent this import and consequently affect peroxisome-dependent metabolic pathways. This results in characteristic accumulations or shortages of metabolites, the degradation or synthesis of which depend on these pathways, such as the accumulation of VLCFAs.²

We report seven unrelated individuals and one half-brother with an apparent dominant ZSD, in whom we identified only one single heterozygous pathogenic variant in *PEX6*, the relative expression of which was increased due to allelic expression imbalance (AEI). The AEI-induced overrepresentation of the mutated *PEX6* protein impairs the function of the *PEX1*-*PEX6* complex, which results in a defective import of peroxisomal proteins and thus causes the clinical manifestations in the affected individuals. AEI-induced overrepresentation of a mutant allele might also play a role in other

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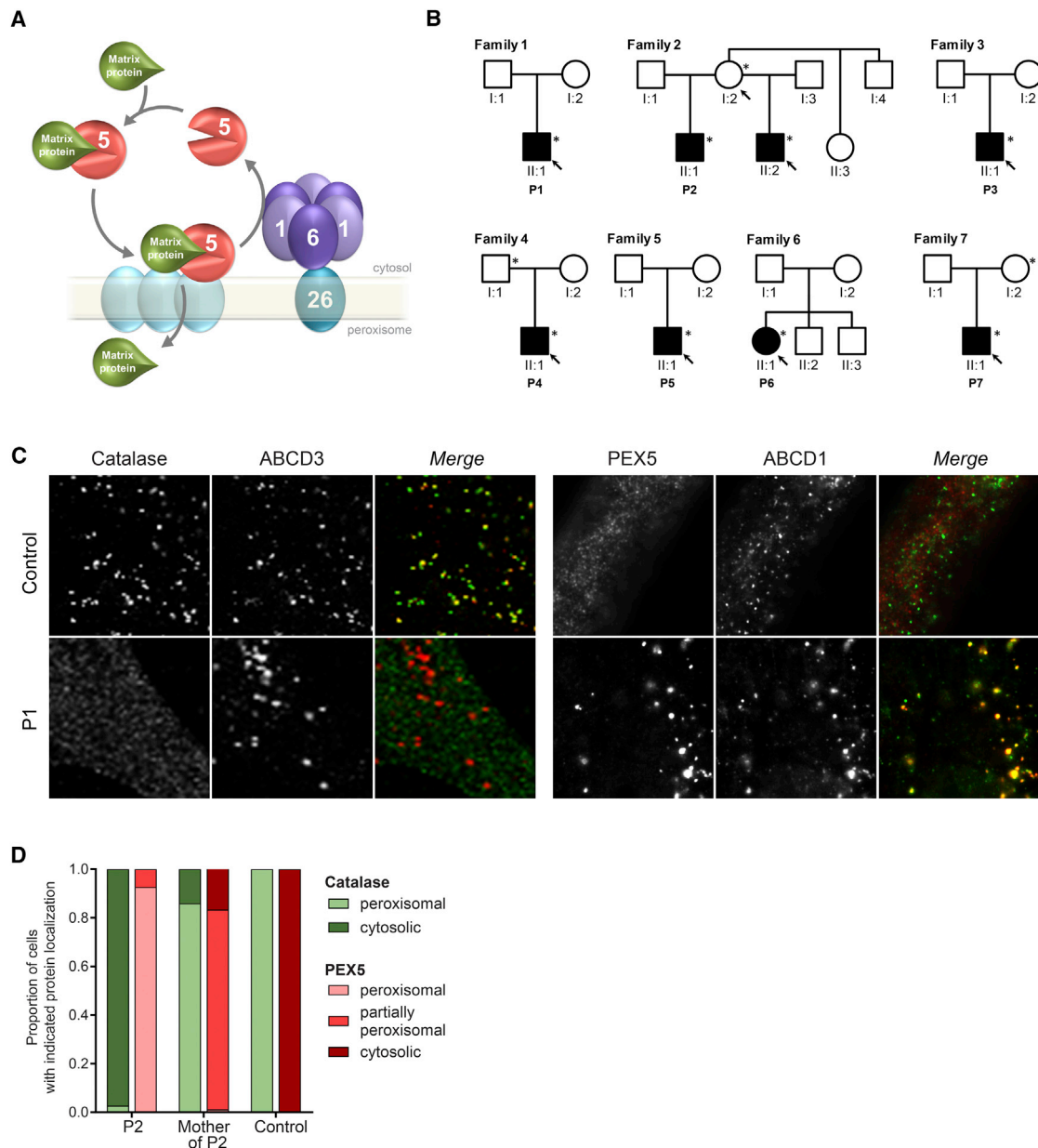


Figure 1. PEX6 Defect in Individuals with ZSD

(A) Schematic presentation of the role of the PEX1-PEX6 complex in the import of peroxisomal matrix proteins into peroxisomes. The peroxisomal protein receptor PEX5 binds matrix proteins, such as catalase, in the cytosol and transports these to the peroxisome, where the receptor docks at PEX proteins in the peroxisomal membrane and releases the matrix proteins into the matrix. After this, PEX5 is exported back into the cytosol through the action of the hexameric PEX1-PEX6 complex, which is anchored to the peroxisome via interaction of PEX6 with PEX26.

(B) Pedigrees of seven affected families with the *PEX6* c.2578C>T variant (only relevant members depicted). Filled symbols represent individuals suffering from a ZSD, asterisks indicate individuals heterozygous for the *PEX6* c.2578C>T variant, and arrows indicate family members from whom primary skin fibroblasts were available for functional studies.

(C and D) Immunofluorescence microscopy assays to determine the subcellular localization of peroxisomal matrix protein catalase (green) and receptor PEX5 (red) together with peroxisomal membrane proteins ABCD3 (red) or ABCD1 (green), respectively.

(C) Representative microscopy images of cells of individual P1 and a control individual. In control cells catalase is peroxisomal (left) and PEX5 cytosolic (right), but in the cells of the affected individuals catalase is mislocalized to the cytosol and PEX5 to peroxisomes, demonstrating a severe defect in the import of the peroxisomal matrix proteins and the export of PEX5 in the cells of affected individuals (see [Figure S1](#) for images of cells from other individuals).

(D) Relative subcellular localization of catalase and PEX5 in cells of individual P2 and his asymptomatic mother, demonstrating only a mild defect in cells of the asymptomatic mother of individual P2 (see also [Figures S1](#) and [S6](#) for additional images).

Table 1. Clinical Characteristics of Affected Individuals and Parents Heterozygous for the PEX6 c.2578C>T (p.Arg860Trp) Variant

	P1 ^a	P2	Half-Brother of P2	Mother of P2	P3 ^b	P4 ^c	Father of P4	P5	P6	P7	Mother of P7	
Subject Characteristics												
Gender	male	male	male	female	male	male	male	male	female	male	female	
Country of origin	the Netherlands	USA	USA	USA	Spain	Sweden	Sweden	Australia	USA	the Netherlands	the Netherlands	
Year of birth	2009	1984	1980	1963	2009	2005	1979	2010	1984	2015	1993	
Age at diagnosis	4 yo	at birth	4 yo	N/A	1 yo	6 yo	N/A	3 yo	2 yo	1 yo	N/A	
Age at death	N/A	8 yo	11 yo	N/A	N/A	10 yo	N/A	N/A	20 yo	N/A	N/A	
Neurological												
Hypotonia	+	+	+	-	+	-	-	+	+	+	-	
Gait abnormalities	+	+	+	-	+	+	-	+	+	-	-	
Developmental delay	+	+	+	-	+	+	-	+	+	-	-	
Intellectual disability	+	ND	+	-	+	+	-	-	+	-	-	
Neuropathy	+	+	+	ND	+	+	-	ND	ND	-	-	
White matter abnormalities	+	ND	+	ND	+	+	-	+	ND	+	ND	
Sensory												
Vision	-	+	+	ND	+	ND	-	-	+	ND	-	
Hearing	+	ND	ND	ND	+	ND	-	+	ND	-	-	
Other												
Hepatomegaly / liver dysfunction	-	+	+	ND	+	ND	-	+	+	+	-	
Adrenal insufficiency	-	ND	ND	ND	+	ND	-	-	ND	-	-	

+, defect present; -, no abnormalities; yo, years old; N/A, not applicable; ND, no data.
^aIndividual P1 additionally displays recurrent calcium oxalate kidney stones from age 7 onwards.
^bIndividual P3 additionally displays cardiac malformation (atrial septal defect, multi-perforated foramen ovale type).
^cIndividual P4 was diagnosed with partial trisomy 18 (SNP array 250k, 18p 0-3423586, hg18, spanning 18p11.32 and parts of 18p11.31).

autosomal-recessive disorders, in which only one heterozygous pathogenic variant is identified.

Material and Methods

Clinical Information

All subjects or their legal representatives provided written informed consent for this study. The procedures followed were in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national). An overview of the main clinical features of the eight affected individuals (seven unrelated individuals and one half-brother) is presented in Table 1. All individuals showed multiple symptoms characteristic for a ZSD. Most individuals had visual impairment and/or sensorineural hearing loss. Liver dysfunction and adrenal insufficiency, both typical for ZSD,² were also frequently noted. All affected individuals showed neurological involvement, including profound hypotonia, gait abnormalities, developmental delay, neuropathy, and white matter abnormalities on brain magnetic resonance imaging (MRI). The clinical courses were progressive without a clear episode of rapid deterioration occurring. Individuals P4, P6, P2, and P2's half-brother died between 8 and 20 years of age, whereas individuals P1, P3, P5, and P7 were still alive at the time of this study.

Biochemical Analysis of Peroxisomal Parameters

We measured very long-chain fatty acid (VLCFA) concentrations and ratios in plasma of family 2 by using capillary gas chromatography of fatty acid methyl esters as previously described.⁷ VLCFA, phytanic, and pristanic acid concentrations in plasma of family 4 were measured as follows. The fatty acids were extracted and methylated in one step using HCl and methanol, before being separated in a gas chromatograph and detected by mass spectrometry using electron ionization and quantified using internal calibrators D3-Phytanic acid, C19:0, and C27:0. Plasma phytanic acid methyl ester of individual P1 and P6 was quantified by capillary gas chromatography⁸ and plasma pipercolic acid was measured by isotope dilution mass-fragmentometry of the t-butyl-dimethylsilyl derivative of pipercolic acid.⁹ VLCFA concentrations and ratios in plasma of family 7 were measured by using UPLC-tandem MS in ESI-negative mode with Waters Micromass Quattro Micro API-tandem MS. VLCFAs in plasma were hydrolyzed with acetonitrile and HCl, extracted with hexane, and concentrated and separated on UPLC with Waters Acquity BEH C18 1.7 μm 2.1 × 50 mm column in mobile phase of acetic acid (no fragmentation of metabolites with similar mass of mother and daughter components). Quantification was done using the stable-isotope dilution technique; deuterated analogs of metabolites (D3-Pristanic acid, D3-Phytanic acid, D4-Docosanoic acid, D4-Tetracosanoic acid, D4-Hexacosanoic

acid) were used as internal standards. Pilocolic acid in plasma of family 7 was measured by using ion-pair-UPLC and detected with a Quattro Premier-LC-MSMS in the multiple reaction monitoring mode (MRM) in the ESI-positive mode (internal standard: D3-L-leucine).¹⁰ We measured VLCFA concentrations and assessed the processing of thiolase in cultured primary skin fibroblasts as previously described.^{11,12}

Genetic Analysis

To perform whole-exome sequencing, DNA was isolated from skin fibroblasts of family 2 and from blood samples of individual P3 using established protocols. Whole-exome sequencing of individuals from family 2 was performed using SureSelect Human All Exon 51 Mb V5 capture kit (Agilent) on a HiSeq 2500 (Rapid Flow Cell V1; Illumina). Whole-exome sequencing of individual P3 was performed using the SeqCap EZ Human Exome Kit v3.0 (Roche Nimblegen) with 100-bp paired-end read sequences generated on a HiSeq2000 (Illumina) in the Centro Nacional de Análisis Genómico in Barcelona (CNAG). Sequences were aligned to hg19 by Burrows-Wheeler Aligner (BWA mem) and single variants and insertions/deletions (indels) were identified through the GATK best practices for Germline SNP & Indel discovery in whole-exome sequencing.

Peroxisome gene panel sequencing was performed with DNA of individuals P3, P4, and P7 using IonTorrent (Thermo Fisher Scientific; included genes: *ABCD1*, *ABCD2*, *ABCD3*, *ACBD5*, *ACOX1*, *ACOX2*, *AGPS*, *AMACR*, *GNPAT*, *HSD17B4*, *PEX1*, *PEX2*, *PEX3*, *PEX5*, *PEX6*, *PEX7*, *PEX10*, *PEX11A*, *PEX11B*, *PEX11G*, *PEX12*, *PEX13*, *PEX14*, *PEX16*, *PEX19*, *PEX26*, *PHYH*, *SCP2*), or with DNA of individual P5 using a 75 *PEX* and related peroxisome gene panel using the Nimblegen SeqCap EZ choice library (Roche) and a paired-end protocol on a MiSeq (Illumina). Sequences were aligned to hg19 with BWA and variations were detected with GATK.¹³

For Sanger sequencing of *PEX6*, genomic DNA was isolated from primary skin fibroblasts using the NucleoSpin Tissue genomic DNA purification kit (Macherey-Nagel). The majority of forward and reverse primers used for sequencing *PEX6* exons, 3' UTR, and potential promoter regions listed in Table S1 were tagged with a -21M13 (5'-TGTAACGACGGCCAGT-3') sequence or M13rev (5'-CAGGAAACAGCTATGACC-3') sequence, respectively. PCR fragments were sequenced using "-21M13," "M13rev" primers, and/or the respective *PEX6* primers, by means of BigDye Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems) and analyzed on an Applied Biosystems 3130x1 or 3730x1 DNA analyzer (Applied Biosystems), following the manufacturer's protocol. Sequence reads (electropherograms) were analyzed using CodonCode Aligner software package (CodonCode Corporation) and compared to *PEX6* reference sequence GenBank: NM_000287.3 (GRCh38, hg38).

For mRNA sequence analysis, we isolated RNA from primary skin fibroblasts of all affected individuals and control individuals using trizol (Invitrogen) extraction and reverse transcribed it into cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN) before Sanger sequencing. For mRNA stability assays, we incubated primary fibroblasts with 1 μ M actinomycin D (ActD) for the indicated time or 20 μ M emetine for 8 hr, before harvesting the cells for RNA isolation.

To assess the expression imbalance of two alleles, the height of the peaks of heterozygous variants in the electropherograms were measured and the peak height ratio of the mutant

peak "mutant/(wild-type+mutant)" calculated, as described previously.¹⁴

Cell Culturing and Transfections

We used primary skin fibroblasts from seven affected individuals and the mother of individual P2, as well as primary skin fibroblast cell lines that are completely deficient of *PEX6* (homozygous for c.402delC [p.Gly135Aspfs*23]¹⁵). Cells were cultured in Dulbecco's modified Eagle's medium with L-glutamine (Bio-Whittaker), supplemented with 10% fetal bovine serum (Bio-Whittaker), 25 mM HEPES buffer (BioWhittaker), 100 U/mL penicillin, 100 μ g/mL streptomycin (LifeTechnologies), and 250 ng/mL Fungizone (LifeTechnologies) in a humidified atmosphere of 5% CO₂ at 37°C or 40°C. Transfection of fibroblasts for immunofluorescence and functional assays was performed using the AMAXA NHDF nucleofector kit (Lonza) following the manufacturer's instructions (program U23). Transfection of HEK-FlpIn cells was performed in 96-well or 6-well plates using the jetPRIME DNA transfection kit (Polyplus transfection) according to the manufacturer's instructions. 24 hr after transfection, the medium was changed and the cells were imaged 48–72 hr after transfection.

Genetic Complementation and Functional Assays

We performed genetic complementation of fibroblasts by transfection of the cells from the affected individuals with *PEX* cDNA, as described in Ebberink et al.³

To test the functional consequences of the *PEX6* variants on peroxisomal protein import, we co-transfected plasmids containing the different *PEX6* variants or pcDNA3 vectors without insert in various ratios (1 versus 3:1 versus 1:1 versus 1:3 versus 0) together with the peroxisomal matrix marker pGFP-SKL¹⁶ into skin fibroblasts deficient for *PEX6* (6-well plates, 2 μ g DNA per transfection in total). The *PEX6* variant constructs pcDNA3-*PEX6* c.821T¹⁷ and pcDNA3-*PEX6* c.2578T were generated by site-directed mutagenesis of the mammalian expression vector pcDNA3 containing full-length *PEX6* cDNA using the QuikChange Site-Directed Mutagenesis Kit from QIAGEN (Hilden) following the manufacturer's instructions. We analyzed the subcellular localization of the fluorescent signal 2 to 3 days after transfection using the fluorescence microscope Zeiss Axio Observer A1. Cells with restored peroxisomal protein import displaying a punctate GFP signal and cells displaying cytosolic GFP signal were quantified by determining the GFP signal in 100–300 cells in three to seven independent experiments.

To test the ability of the *PEX6* variants to localize the *PEX1/6* complex to peroxisomes, we transfected pcDNA3-*PEX1*, pcDNA3-*PEX6* and/or pcDNA3-*PEX6* c.2578T into skin fibroblasts deficient for *PEX6* before performing immunofluorescence assays.

To test the effect of miRNA inhibitors, which were predicted to target the longer *PEX6* c.*1_462 mRNA by TargetScan and microRNA.org, on the *PEX6* allelic expression, we co-transfected skin fibroblasts derived from the affected individuals with GFP-SKL and miRIDIAN microRNA Human hsa-miR-150-5p ("miR150") and hsa-miR-33a-5p ("miR33") Hairpin Inhibitor or Hairpin Inhibitor Negative Control #1 (50–70 nM, Dharmacon). We determined the subcellular localization of GFP signal by fluorescence microscopy and quantified the number of peroxisome-positive fibroblasts after anonymizing samples for unbiased assessment after 2 to 7 days. Additionally, we isolated the RNA from the fibroblasts and performed quantitative RT-PCR to measure the mRNA levels of *PEX6*, long *PEX6* c.*1_462, and miR33

and miR150 target genes *CPT1a* and *p53* 2 to 4 days after transfection.

Quantitative RT-PCR

We isolated RNA from primary fibroblasts and reverse transcribed it to cDNA for quantitative RT-PCRs. We measured the mRNA levels of target genes in duplicates using the SensiFAST SYBR No-ROX Kit (Bioline) and the Lightcycler 480, Instrument II (Roche). The N_0 values of target genes were normalized to the geometric mean of the reference genes *Nono* and *hsH3F3A*, which were determined most stable in expression and thus best suited for normalization out of ten assessed reference genes (determined with Normfinder algorithm¹⁸).

Immunofluorescence Imaging

We analyzed the peroxisomal phenotype in skin fibroblasts by confocal immunofluorescence microscopy. The cells were cultured on glass slides to a confluency of 50%–80%. For fixation, we treated the cells with 2% paraformaldehyde (Merck) in PBS for 20 minutes at room temperature and permeabilized with 0.5% Triton X-100 (Bio Rad) for 5 min. We used primary antibodies against the peroxisomal matrix protein catalase (monoclonal Mab 17E10, own production), the peroxisomal membrane protein ABCD3 (PMP70, rabbit, #71-8300, Thermo Fisher Scientific), PEX1 (mouse, # 611719, BD Biosciences), and PEX5 (rabbit, gift from G. Dodt) and as secondary antibodies either biotinylated α -mouse antibodies (E 433; Dako) and streptavidin-FITC (F 422; Dako) or Alexa Fluor 555 goat anti-rabbit (Invitrogen). The slides were fixed on mounting medium Vectashield H1000 (Brunschwig). Images were taken using the fluorescence microscope Zeiss Axio Observer A1 (100 \times magnification) or the Leica TCS-SP8 filter-free Spectral Confocal Microscope (63 \times magnification) and processed with the Leica Application Suite AF Lite software version 2.6.3 (Leica). If required, brightness and contrast of whole images were adjusted using the Adobe Photoshop CS6 software (Adobe Systems).

Immunoblotting

We performed immunoblot analyses using whole-cell lysates, of which proteins were separated by SDS polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane using a semidry blotting process. Subsequently, we used primary antibodies against PEX6 (HPA025924, Sigma-Aldrich), peroxisomal thiolase (3-ketoacyl-CoA thiolase, HPA007244, Atlas antibodies), α -tubulin (T6199, Sigma-Aldrich), or β -actin (A5441, Sigma-Aldrich) and secondary antibodies IRDye 800CW Goat anti-Rabbit IgG (LI-COR Biosciences) or IRDye 680RD Goat anti-Mouse IgG (H+L; LI-COR Biosciences) with the Odyssey Imaging System (LI-COR Biosciences).

NanoLuc Assay

We constructed NanoLuc expression vectors by cloning the *PEX6* 3' UTR sequence c.*1_*650 wild-type or with the deletion c.*442_445delTAAA (GeneScript) into the pNL3.2[NlucP/minP] vector (Promega). Correct cloning was confirmed by Sanger sequencing of the vectors. We mutated the proximal polyadenylation signal by introducing a c.*308A>C change, creating the vector pNluc_*PEX6* c.*308C, by site-directed mutagenesis of the pNluc-wild-type vector using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). We integrated the NanoLuc-*PEX6* 3' UTR constructs into HEK-FlpIn cells and confirmed the stable NanoLuc expression by quantitative RT-PCR. Additionally, we confirmed

that NanoLuc-*PEX6* c.*308C was transcribed as NanoLuc mRNA with the longer *PEX6* c.*1_*462 3' UTR and that NanoLuc-*PEX6* c.*442_445delTAAA was transcribed as NanoLuc mRNA with the shorter *PEX6* c.*1_*326 3' UTR using the rapid amplification of cDNA ends (RACE) method. We transfected 50 nM miRIDIAN microRNA Mimic Negative Control #1 or miRIDIAN microRNA Human hsa-miR-33a-5p, hsa-miR-33b-5p, or hsa-miR-150-5p Mimics (Dharmacon) into the HEK-FlpIn-Nanoluc-*PEX6* c.*308C cells and HEK-FlpIn-Nanoluc-*PEX6* c.*442_445delTAAA cells. 24 hr after transfection, we used the Nano-Glo Dual-Luciferase Reporter Assay System (Promega) to measure luminescence at three subsequent time points. To validate miRNA efficiency, we confirmed the downregulated expression of miRNA target genes *CPT1a* or *cMyb*, respectively, using quantitative RT-PCR.

SV40 Immortalized Cell Model with Stable *PEX6* Overexpression

We generated *PEX6* expression vectors containing wild-type *PEX6* cDNA (pMono-hygro-*PEX6*) or mutant *PEX6* c.2578T cDNA (pMono-neo-*PEX6*_c.2578T) by cloning wild-type *PEX6* c.2578C or mutant *PEX6* c.2578T from pcDNA3 vectors into pMono-hygro-mcs or pMono-neo-mcs (InvivoGen), using restriction enzymes with compatible ends (i.e., BspEI/XbaI and AgeI/AvrII, respectively) and then transfected them into SV40 immortalized human control fibroblasts. After selection with the respective antibiotics (100–200 μ g/mL geneticin, 150 μ g/mL hygromycin B), we confirmed the overexpression of *PEX6* mRNA by immunoblot analysis, quantitative RT-PCR (see primers in Table S1) and—in case of pMono-neo-*PEX6* c.2578T—by Sanger sequencing.

Statistical Methods

All values in figures are presented as the mean \pm SD. Results were analyzed with Mann-Whitney U tests or unpaired t tests, in case of mean allele peak ratios, with one-sample t tests with a theoretical mean of 0.5. A p value less than 0.05 was considered statistically significant. Statistical analyses were carried out in the Graphpad Prism 6 software.

Results

Individuals with ZSD Display Same Heterozygous *PEX6* Mutation

The seven unrelated individuals (Figure 1B) were diagnosed with a ZSD on the basis of their clinical symptoms (Table 1), aberrant peroxisomal metabolite levels in blood and fibroblasts (Tables 2 and S2), and an import defect of peroxisomal matrix proteins in fibroblasts (Figures 1C and S1).

By means of functional genetic complementation of cultured primary skin fibroblasts³ (of individuals P1, P3, and P4) or fused cell complementation¹⁹ (of individual P6), we identified a defective *PEX6* gene as the cause of the ZSD in four affected individuals. When we subsequently Sanger sequenced *PEX6* (GenBank: NM_000287.3) to identify the disease-causing mutations, we detected only the heterozygous variant c.2578C>T (p.Arg860Trp) (rs61753230) in all four individuals, but no second potentially pathogenic variant (Table 3). The same single heterozygous *PEX6* c.2578C>T variant was also independently

Table 2. Peroxisomal Parameters in Primary Skin Fibroblasts Derived from Affected Individuals and Parents Heterozygous for the PEX6 c.2578C>T (p.Arg860Trp) Variant

	Individual	P1	P2	Mother of P2	P3	P4	P5	P6	P7	Reference Range
	Age at sampling	3 yo	1 yo	21 yo	1 yo	8 yo	ND	ND	1 yo	
VLCFAs concentration (in $\mu\text{mol/g}$ protein)	C22:0	3.13	4.2	5.31	1.19	3.4	4.02	2.88	2.19	2.46–6.59
	C24:0	7.56	11.05	11.81	6.06	8.18	10.46	8.91	8.29	6.37–13.87
	C26:0	0.95	1.19	0.41	1.39	0.61	1.51	1.18	1.35	0.16–0.41
VLCFA ratios	C24:0/C22:0	2.42	2.63	2.22	3.17	2.4	2.6	3.1	3.78	1.68–2.92
	C26:0/C22:0	0.3	0.28	0.08	0.73	0.18	0.38	0.41	0.62	0.03–0.1
Thiolase immunoblot ^a	44 kDa	+/-	+/-	-	+/-	+/-	+/-	+/-	-	-
	41 kDa	+/-	+/-	++	+/-	+/-	+/-	+/-	+/-	++
Catalase import deficiency ^b	[percentage of deficient cells]	>90%	>90%	<20%	>90%	>90%	>90%	>90%	90%	0%

Abbreviations are as follows: VLCFAs, very long-chain fatty acids; yo, years old; ND, no data.

^aFor assessment of peroxisomal processing of thiolase

^bCompare Figure S1

identified by Sanger sequencing in individual P2 and his half-brother and in individuals P5 and P7. Peroxisome-specific gene-panel sequencing in four individuals (P3, P4, P5, and P7) and whole-exome sequencing in two individuals (P2 and P3) did not identify additional pathogenic variants in other genes encoding peroxisomal proteins that could explain the phenotype of the individuals.

PEX6 Mutation Impairs Peroxisome Biogenesis, but Not PEX6 Protein Localization

The mutated arginine-860 residue constitutes the arginine finger 2 of PEX6, which is a highly conserved residue in AAA+ ATPases and located in the second region of homology (SRH) of the ATP-binding D2 domain in close vicinity to bound ATP (Figure S2).^{20,21} The PEX6 c.2578C>T variant is absent in genome databases of healthy populations or affected individuals, including ExAC and ClinVar, but was reported once as a single heterozygous variant in an individual diagnosed with ZSD.²² The variant is predicted as deleterious by SIFT²³ (score 0), disease causing by MutationTaster²⁴ (score 1.0), and probably damaging by PolyPhen-2²⁵ (score 1.0). We confirmed the predicted pathogenicity of the variant by expressing PEX6 p.Arg860Trp in PEX6-deficient skin fibroblasts, which, in contrast to wild-type PEX6, did not restore peroxisomal matrix protein import (Figure S3).

To study the functional consequences of the PEX6 p.Arg860Trp variant, we assessed PEX6 protein levels in cell lysates of the affected individuals by immunoblot analysis, but this did not show marked changes when compared to control fibroblasts (Figure S4). Moreover, immunofluorescence microscopy showed that the PEX1-PEX6 complexes in fibroblasts of the affected individuals were correctly localized at peroxisomes (Figure S5). This observation corroborates our finding that expression of PEX6 p.Arg860Trp in PEX6-deficient cells also resulted in correct peroxisomal localization of PEX1-PEX6 complexes (Figure S5). PEX5, on the other hand, which is

predominantly cytosolic in control cells, was mainly localized at peroxisomal membranes in cells of the affected individuals (Figure 1C). These observations indicate that PEX6 p.Arg860Trp results in defects in PEX5 export and peroxisome biogenesis in cells of the affected individuals, although it is correctly localized (Figure 1C).

Asymptomatic Parents with PEX6 Mutation Show Only Very Mild Peroxisomal Defects

We next Sanger sequenced PEX6 of the parents of the affected individuals, which revealed that individuals P1 and P3 obtained the variant *de novo* (biological parenthood confirmed). Individual P2 and his half-brother inherited the variant from their mother, individual P4 from his father, and individual P7 from his mother (Table 3). For the other individuals, parental DNA was not available.

All three parents who were heterozygous for the PEX6 c.2578C>T variant showed no clinical symptoms or any signs that could be related to a peroxisomal defect (Table 1) and, in contrast to the affected individuals, only minor biochemical abnormalities in blood and fibroblasts (Tables 2 and S1). Furthermore, we observed that PEX5 was only partly mislocalized to peroxisomes in fibroblasts of the mother of individual P2, who was the only parent heterozygous for the PEX6 c.2578C>T variant of whom cells were available for analysis (Figures 1D and S6). This slight PEX5 export defect corresponded to a very mild import defect of the peroxisomal matrix protein catalase in these cells (Figures 1D and S1). Based on these observations we considered a classical dominant-negative disease mechanism in the affected individuals (due to mere heterozygosity of the pathogenic PEX6 c.2578C>T variant) unlikely.

Affected and Control Individuals with a Heterozygous 3' UTR Variant Show Allelic Expression Imbalance of PEX6

Interestingly, when we analyzed PEX6 mRNA expression by Sanger sequencing cDNA derived from the affected individuals' fibroblasts, we noted that the levels of the mutant

Table 3. Genomic PEX6 Variants in Affected Individuals and Parents Heterozygous for the PEX6 c.2578C>T (p.Arg860Trp) Variant

PEX6 Polymorphism		Alleles in Individuals													
refSNP Cluster ID	DNA Sequence Change ^a	Amino Acid Change	Minor Allele Frequency (1000 Genomes)	P1	P2	Mother of P2	P3	P4	Father of P4	Mother of P4	P5	P6	P7	Father of P7	Mother of P7
rs9462859	c.1_55C>T	-	A = 0.334 / 1672	T/C	T/C	T	T/C	T/C	T	T/C	T/C	T/C	T/C	C	T
rs9462858	c.279G>A	p.Arg93Arg		G	G	G/A	G	G	G	G	G	G	G	G	G
rs61753220	c.399G>T	p.Val133Val	A = 0.332/1662	T/G	T/G	T	T/G	T/G	T	T/G	T/G	T/G	T/G	G	T
rs9986447	c.853C>G	p.Pro285Ala	C = 0.01/3 ^b	C	C	C	C	C	C	C	C	C/G	C	C	C
rs61753230	c.883-3T>C	-	G = 0.29/1451	C/T	C/T	C	C/T	C/T	C	C/T	C/T	C/T	C/T	T	C
rs1129186	c.2578C>T	p.Arg860Trp		C/T	C/T	C/T	C/T	C/T	C/T	C	C/T	C/T	C/T	C	C/T
rs1129187	c.2814G>A	p.Glu938Glu	T = 0.494/2472	A/G	A/G	A	A/G	A/G	A	A/G	A/G	A/G	A/G	G	A
rs144286892	c.2816C>A	p.Pro939Gln	T = 0.332/1660	A/C	A/C	A	A/C	A/C	A	A/C	A/C	A/C	A/C	C	A
	c.*442_445delTAAA	-	T = 0.353/1766	TAAA / delTAAA	TAAA / delTAAA	delTAAA / delTAAA	TAAA / delTAAA	TAAA / delTAAA	delTAAA / delTAAA	TAAA / delTAAA	TAAA / delTAAA	TAAA / delTAAA	TAAA / delTAAA	TAAA / delTAAA	TAAA / delTAAA

n/a, not assessed.
^aNucleotide numbering based on GenBank: NM_000287.3, GRCh38/hG38 (on reverse DNA strand)
^bClinical significance (ClinVar) of polymorphism c.853C>G (p.Pro285Ala): likely benign/uncertain significance

PEX6 c.2578T cDNA were consistently three to five times higher than those of wild-type PEX6 (c.2578C) cDNA (Figures 2A and 2B). In contrast, the cDNA levels of both PEX6 alleles were equal in the mother of individual P2. When we Sanger sequenced the PEX6 cDNAs of 22 different fibroblast cell lines derived from unrelated control individuals, we noted AEI of PEX6 in over 40% of the cell lines. This indicates that the AEI is not specific for the cells of the affected individuals but a common feature of PEX6.

To find an explanation for this AEI of PEX6, we compared the coding sequences and 1,000 bp 5' of the start and 500 bp 3' of the stop codon of PEX6 of all affected individuals to those of the parents, who were heterozygous for the PEX6 c.2578C>T variant (mothers of individuals P1 and P7, father of individual P4). This revealed several common variants that were heterozygous in the affected individuals but homozygous in the parents, and thus could be responsible for the AEI (Table 3). Of these, we found the c.1_55C>T (rs9462859), c.399G>T (rs9462858), c.883-3T>C (rs9986447), c.2814G>A (rs1129186), and c.2816C>A variants (rs1129187) also heterozygous in some of the control cells that did not display AEI of PEX6, and consequently excluded these as candidates (data not shown). Heterozygosity for the c.*442_445delTAAA variant in the 3' UTR region of PEX6 (rs144286892), however, always correlated with AEI of PEX6 (Figure 2C). Long-range PCR and clone sequencing confirmed that the PEX6 c.*442_445delTAAA variant was allelic to the PEX6 c.2578T mutation (data not shown).

The PEX6 c.*442_445delTAAA variant is a frequently occurring polymorphism with a minor allele frequency of 0.35 (1000 Genomes). According to the APASdb database, it disrupts the most distal of the two known polyadenylation signals of PEX6, which are located at c.*305_310 and c.*440_445. Usage of the two polyadenylation signals results in PEX6 mRNA with a shorter (PEX6 c.*1_326) or longer (PEX6 c.*1_462 mRNA) 3' UTR, respectively. Quantitative analysis of PEX6 mRNA expression in fibroblast cell lines with different PEX6 allele combinations indeed confirmed that the PEX6 c.*442_445delTAAA allele does not produce the longer PEX6 c.*1_462 mRNA (Figure 2D). Nevertheless, the total levels of PEX6 mRNA expressed by the PEX6 c.*442_445delTAAA allele were consistently higher than those expressed by the allele without the deletion (Figure 2D). This expression level difference of the two alleles explains why AEI of PEX6 occurs in all cell lines heterozygous for PEX6 c.*442_445delTAAA, but not in the cell lines homozygous for any of the PEX6 alleles (Figure 2C).

AEI Is Not Caused by Differences in Stability of Longer or Shorter PEX6 mRNA

To determine whether the relatively increased levels of the shorter PEX6 c.*1_326 mRNA, which is expressed by the PEX6 c.*442_445delTAAA allele, may be due to a decreased stability of the longer PEX6 c.*1_462 mRNA, we treated cells of the affected individuals with the nonsense-mediated

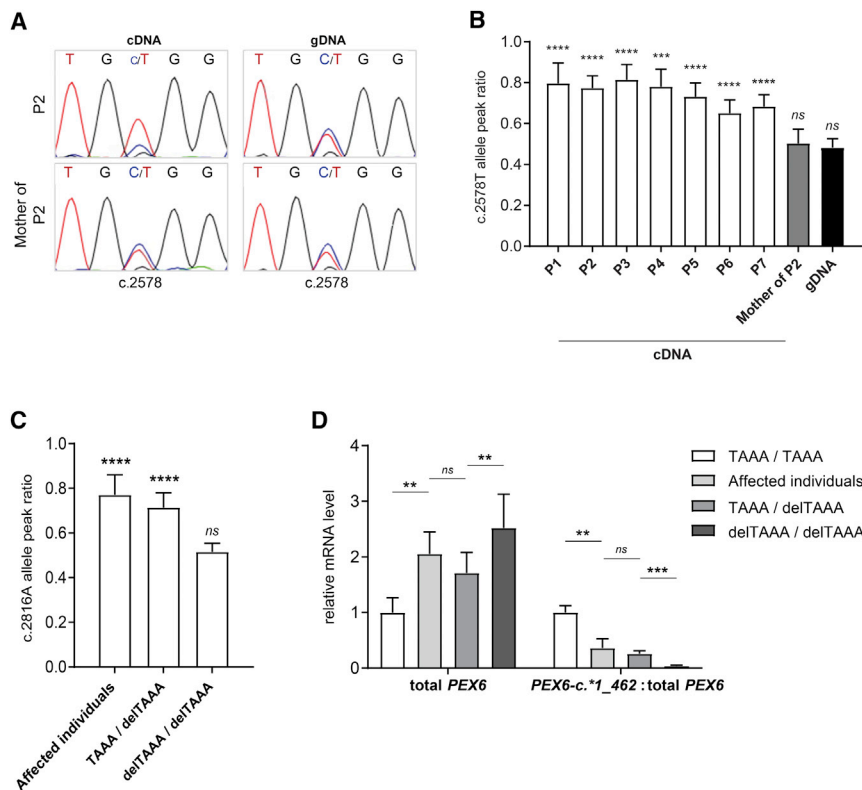


Figure 2. AEI of *PEX6* in Affected Individuals and Control Individuals with the Heterozygous 3' UTR Variant *PEX6* c.*442_445delTAAA

(A and B) Allelic expression of *PEX6* mRNA in cells of affected individuals and the mother of affected individual 2. *PEX6* cDNA and genomic DNA were Sanger sequenced and the peak height of both *PEX6* alleles were used to determine the relative abundance of each allele (see representative sequence reads in A). Mean c.2578T allele peak ratios in the different cell lines (B), expressed as the ratio of the mutant allele peak height (c.2578T) over the total peak height (c.2578T+c.2578C) as described in Moncini et al.,¹⁴ depicted as mean with standard deviation of 6–19 analyzed electropherograms per cell line (or gDNA group) using 2–3 different primer sets. While the *PEX6* allele ratio is in balance in genomic DNA of the affected individuals and genomic DNA and cDNA of the mother of individual P2 (i.e., equal peak heights), the mutant allele ratio in cDNA of all affected individuals is significantly increased, demonstrating the AEI of *PEX6*. (C and D) *PEX6* mRNA expression in fibroblasts of affected individuals and fibroblasts of 22 unrelated control individuals with different combinations of the *PEX6* c.*442_445delTAAA allele (homozygous for the allele without the deletion ["TAAA / TAAA"], homozygous for the *PEX6* c.*442_445delTAAA allele ["delTAAA / delTAAA"] or compound heterozygous ["TAAA / delTAAA"]).

(C) Mean allele peak ratios for the *PEX6* c.*442_445delTAAA allele in cDNA of cells of the affected individuals and control cells heterozygous and homozygous for this allele, confirming that the AEI of *PEX6* occurs only in the heterozygous cells. Shown are the peak ratios of the c.2816A variant, which is a common polymorphism (MAF 0.39 [ExAc] / 0.33 [1000 Genomes]) that is in *cis* with *PEX6* c.*442_445delTAAA (see also Table 3). The peak ratios were determined as described in (B) and Moncini et al.¹⁴ and are presented as mean with standard deviation based on analysis of 5–10 electropherograms from 4–7 different cell lines per allele combination.

(D) Results of quantitative RT-PCR experiments, demonstrating an increased level of total *PEX6* mRNA but no expression of longer *PEX6* c.*1_462 mRNA in cells homozygous for the *PEX6* c.*442_445delTAAA. Depicted are the *PEX6* mRNA levels normalized to reference gene expression or as ratio *PEX6* c.*1_462 over total *PEX6*, with "TAAA / TAAA" values set as 1, as mean with standard deviation determined in 4–13 cell lines per allele combination.

Statistical analyses for (B) and (C) were performed using one-sample t tests with a theoretical mean of 0.5, and for (D) using Mann-Whitney U tests (**p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, ns not significant).

mRNA decay-inhibitor emetine or the transcriptional inhibitor actinomycin D. We did not observe increased degradation nor decreased stability of the longer *PEX6* c.*1_462 mRNA (Figure S7). Furthermore, we could not find indications that the stability of the longer *PEX6* c.*1_462 mRNA is negatively regulated by the microRNAs hsa-miR-33-5p and hsa-miR-150-5p, both of which are predicted to target sequences present in the extended 3' UTR of the longer *PEX6* c.*1_462 mRNA by TargetScan and microRNA.org (Figures S8 and S9). These results indicate that differential stability of the two *PEX6* mRNA isoforms is not the underlying reason for the AEI.

Overrepresentation of Mutant *PEX6* Impairs Wild-Type *PEX6* Function and Causes Peroxisome Biogenesis Defect

We hypothesized that the peroxisome biogenesis defect in the affected individuals is caused by the AEI-induced overrepresentation of the pathogenic *PEX6* p.Arg860Trp

variant. To confirm this, we overexpressed *PEX6* p.Arg860Trp in SV40-immortalized control fibroblasts that express endogenous *PEX6* (Figure 3A). This resulted in an accumulation of *PEX6* at the peroxisomal membranes and a concomitant defect of catalase import into peroxisomes (Figures 3B and S10). Moreover, when we co-expressed *PEX6* p.Arg860Trp and wild-type *PEX6* in different ratios in *PEX6*-deficient fibroblasts, we observed a significant negative correlation between the restoration of peroxisomal matrix protein import and the ratio of *PEX6* p.Arg860Trp over wild-type *PEX6*. This negative effect on peroxisomal import was far more pronounced than in cells co-expressing wild-type *PEX6* with the previously characterized recessive variant, *PEX6* p.Pro274Leu¹⁷ (Figure 3C). These results indicate that the *PEX6* p.Arg860Trp variant has a negative effect on *PEX1-PEX6* complex function but results in a disease phenotype only when *PEX6* p.Arg860Trp is at least two to three times more abundant than wild-type *PEX6*.

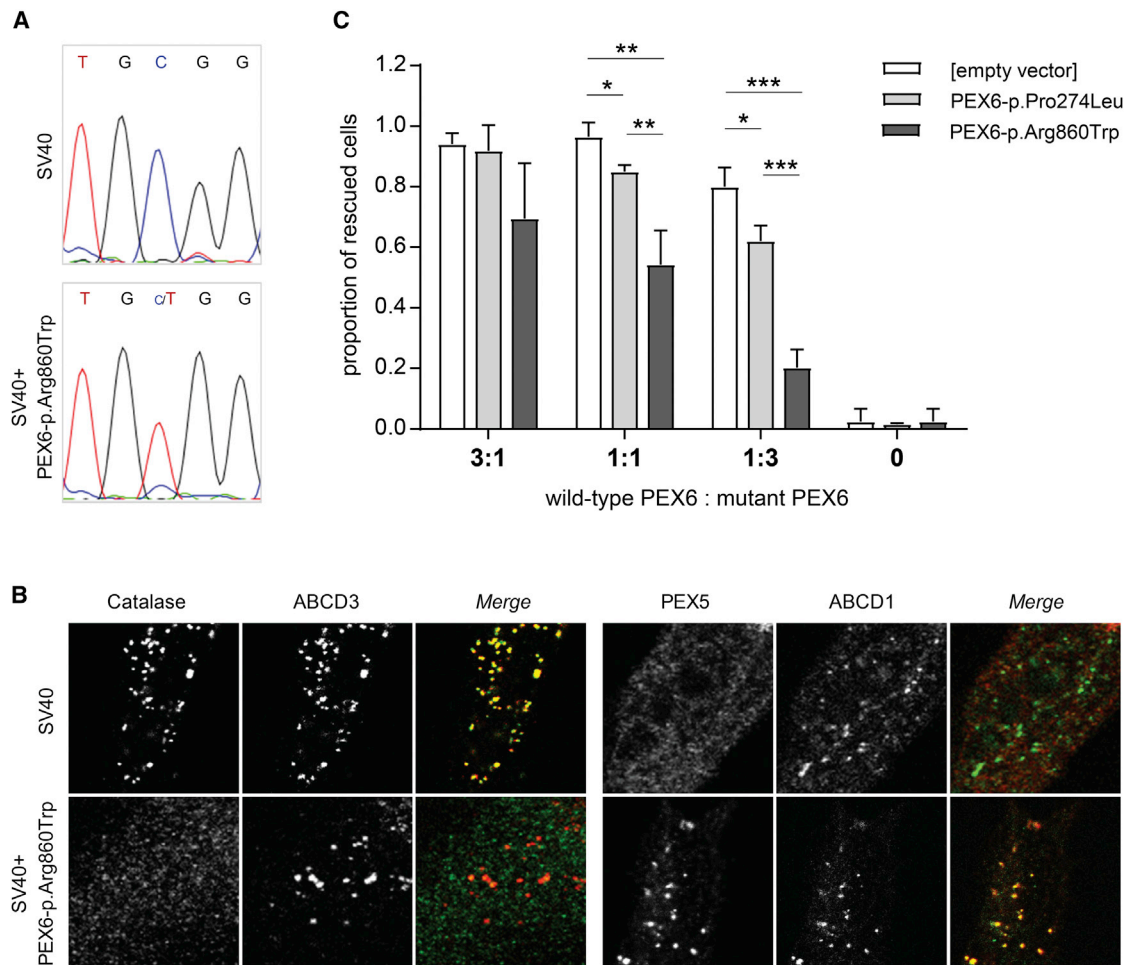


Figure 3. Overexpression Models of Dominant-Negative PEX6-p.Arg860Trp in Presence of Wild-Type PEX6

(A and B) Peroxisome biogenesis in SV40 immortalized control cells overexpressing PEX p.Arg860Trp.

(A) Representative sequence reads obtained by Sanger sequencing of *PEX6* cDNA in SV40 cells and SV40 cells over-expressing *PEX6* c.2578C>T. The mutant *PEX6* c.2578T allele displays a similar overrepresentation when compared to the endogenous wild-type *PEX6* allele as observed in cells of the affected individuals (compare Figure 2A).

(B) Immunofluorescence microscopy images of cells, which were double labeled to determine the subcellular localizations of catalase (green) and ABCD3 (red), or PEX5 (red) and ABCD1 (green), demonstrating a defect in peroxisomal catalase import and PEX5 export in SV40 cells over-expressing *PEX6* p.Arg860Trp (compare also Figure S10).

(C) Co-expression of mutant and wild-type *PEX6* in *PEX6*-deficient cells. *PEX6*-deficient cells were co-transfected with wild-type *PEX6* cDNA and *PEX6* variants *PEX6* c.2578T (p.Arg860Trp) (dark grey) or *PEX6* c.821T (p.Pro274Leu) (light grey) in different ratios, together with the peroxisomal matrix protein marker GFP-SKL, whereupon the degree of GFP-SKL import into peroxisomes was determined. GFP-SKL import after transfection with wild-type *PEX6* only was set as 1. Increased relative expression of *PEX6*-p.Arg860Trp results in a decreased peroxisomal import of GFP-SKL, which was far more pronounced than observed with *PEX6* p.Pro274Leu.

Data are depicted as mean with standard deviation of three independent experiments counting 180–300 cells per condition, and statistical analyses were performed using unpaired t tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, while an absence of a p value indicates non-significance).

Discussion

We identified AEI-induced overrepresentation of a pathogenic *PEX6* c.2578C>T allele as the cause of disease in seven unrelated individuals and one half-brother with an apparent dominant ZSD. The fact that the same *PEX6* c.2578C>T mutation occurred in seven unrelated families, among which two individuals in which the mutation arose *de novo*, may be related to the fact that c.2578C is part of a CpG site, which is prone to mutations.²⁶ *De novo* occurrence of *PEX* mutations have been reported previously.²⁷

Four of the affected individuals inherited the *PEX6* c.2578C>T allele from one of the parents, who in all cases were asymptomatic and did not show AEI of *PEX6*.

We showed that the *PEX6* p.Arg860Trp change has a dominant-negative effect on the function of the PEX1-*PEX6* complex in peroxisomal matrix protein import. However, as observed in the parents, heterozygosity for the *PEX6* c.2578C>T allele does not result in a disease phenotype when this allele is equally expressed as the wild-type allele, but only when overrepresented due to AEI. The AEI is correlated with heterozygosity for a

common *PEX6* c.*442_445delTAAA deletion in the 3' UTR of *PEX6*. This deletion eliminates the most distal of two known polyadenylation sites of *PEX6*, resulting in the exclusive and increased expression of the *PEX6* mRNA with a shortened 3' UTR (*PEX6* c.*1_326). mRNAs with shorter 3' UTRs have been described previously to be higher expressed than their counterpart with a longer 3' UTR, which often has been attributed to differences in their mRNA stability.^{28,29} We have not been able to elucidate the cause for the observed AEI of *PEX6*, but excluded enhanced degradation and decreased stability of the longer mRNA. This AEI might thus possibly be caused by differences in gene transcription or mRNA processing of the longer and shorter *PEX6* isoform.

The arginine at position 860 constitutes the arginine finger 2 in the D2 ATPase domain of *PEX6*, which is a highly conserved domain in AAA+ ATPases (Figure 2).^{20,21} Previous studies have shown that ATP binding in this domain is essential for the physical interaction of *PEX6* with both *PEX1* and the peroxisomal membrane anchor *PEX26* (Figure 1A).⁵ The *PEX5* export by the *PEX1*-*PEX6* complex requires hydrolysis of the bound ATP.^{4,5} Our finding that the *PEX1*-*PEX6* p.Arg860Trp complex still localizes to peroxisomes but is incapable of *PEX5* export implies that the mutation does not affect ATP binding in D2 but prevents ATP hydrolysis. In line with this, mutations of arginine finger 2 in the two double-ring AAA+ ATPases NSF and p97 have also been reported to abrogate ATP hydrolysis without affecting complex formation and to have a dominant-negative effect on complex function.^{30,31}

Our study reports a dominant presentation of an autosomal-recessive disorder due to AEI promoting the overrepresentation of a pathogenic allele. In autosomal-dominant disorders and several forms of cancer, AEI of various genes has been implicated as risk factors or as modifiers of disease severity.^{14,32–35} Given the high prevalence and genome-wide occurrence of AEI,^{36–38} this disease mechanism should be considered as a possible underlying cause of other autosomal-recessive disorders, in which only single heterozygous mutations are found. Thus, our findings bear great relevance for the interpretation of whole-exome and genome sequence data in clinical diagnostic laboratories, where allelic expression levels are not functionally verified and heterozygous variants inherited from asymptomatic parents are usually filtered out or considered non-pathogenic. Finally, similar as described for autosomal-dominant disorders, AEI may modulate the phenotypic variability in compound heterozygous individuals with autosomal-recessive disorders.

Supplemental Data

Supplemental Data include ten figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.ajhg.2017.11.007>.

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Web Resources

1000 Genomes, <http://www.internationalgenome.org/>
APASdb, <http://genome.bucm.edu.cn>
Burrows-Wheeler Aligner, <http://bio-bwa.sourceforge.net/>
ClinVar, <https://www.ncbi.nlm.nih.gov/clinvar/>
dbSNP, <https://www.ncbi.nlm.nih.gov/projects/SNP/>
ExAC Browser, <http://exac.broadinstitute.org/>
GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>
miRNA.org, <http://www.microRNA.org>
OMIM, <http://www.omim.org/>
RCSB Protein Data Bank, <http://www.rcsb.org/pdb/home/home.do>
TargetScan 6.2, <http://www.targetscan.org/>
UniProt, <http://www.uniprot.org/>

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