Functional analysis of PEX13 mutation in a Zellweger syndrome spectrum patient reveals novel homooligomerization of PEX13 and its role in human peroxisome biogenesis

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In humans, the concerted action of at least 13 different peroxisomal PEX proteins is needed for proper peroxisome biogenesis. Mutations in any of these PEX genes can lead to lethal neurometabolic disorders of the Zellweger syndrome spectrum (ZSS). Previously, we identified the W313G mutation located within the SH3 domain of the peroxisomal protein, PEX13. As this tryptophan residue is highly conserved in almost all known SH3 proteins, we investigated the pathogenic mechanism of the W313G mutation and its role in PEX13 interactions and functions in peroxisome biogenesis. Here, we report for the first time that human PEX13 interacts with itself in peroxisomes in living cells. We demonstrate that the import of PTS1 (peroxisomal targeting signal 1) proteins is specifically disrupted when homooligomerization of PEX13 is interrupted. Live cell FRET microscopy in living cells as well as co-immunoprecipitation experiments reveal that the highly conserved W313 residue is important for self-association of PEX13 but is not required for interaction with PEX14, a well-established interaction partner at the peroxisomal membrane. Experiments with truncated constructs indicate that although the W313G mutation resides in the C-terminal SH3 domain, the N-terminal half is necessary for peroxisomal localization, which in turn appears to be crucial for homooligomerization. Furthermore, rescue of homooligomerization in the W313G mutant cells through complementation with truncation constructs restores import of peroxisomal matrix proteins. Taken together, the thorough analyses of a ZSS patient mutation unraveled the general cell biological function of PEX13 and its mechanism in the import of peroxisomal matrix PTS1 proteins.

INTRODUCTION

In the past years, peroxisomes have gained increasing attention as they encompass diverse cellular mechanisms in mammals including the breakdown of very long chain fatty acids and branched chain fatty acids, as well as scavenging peroxides and reactive oxygen species. Peroxisomes also provide crucial enzymatic steps in the synthesis of plasmalogens, which are ether-linked phospholipids found abundantly in brain and tissues of the nervous system (1). Moreover, recent studies in human hepatocytes and mouse embryonic fibroblasts have revealed that the role of peroxisomes is not restricted to metabolic pathways. They are also signaling sites that promote a rapid immune response to viral infection (2). In plants, β-oxidation of fatty acids from seed oil takes place exclusively in the peroxisomes, and accessing this stored carbon is essential for seedling development following germination (3). The glycosome, a member of the peroxisome family found in several Trypanosomatidae species responsible for serious tropical diseases of humans, has received particular interest since proper localization of matrix proteins in the glycosome has been demonstrated to be essential for survival of the parasites (4,5).

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The growing spectrum of peroxisomal single-enzyme deficiencies and peroxisome biogenesis disorders (PBDs), including devastating neurological diseases such as Zellweger syndrome spectrum (ZSS; OMIM #601789), demonstrates the importance of the peroxisomal functions in humans [reviewed in (6)]. The genetic complementation of defects in the peroxisome biogenesis in PBD patient fibroblasts or mutants from Chinese hamster ovary (CHO) cells, as well as several yeast species, including Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha and Yarrowia lipolytica, has led to the identification of proteins called peroxins (PEX), which are essential for the peroxisome assembly and function. At present, 13 PEX genes are known in humans and 33 PEX genes in yeasts. The PEX proteins play roles in multiple processes including membrane formation, the insertion of peroxisomal membrane proteins, the import of peroxisomal matrix proteins and fission and degradation of organelles. Peroxins are unique among membrane-bound organelles as they can import not only folded proteins but even oligomeric protein complexes (7,8). Import of peroxisomal matrix proteins requires an intricate orchestration of cytoplasmic and peroxisomal events since peroxisomal proteins must be recognized by cytosolic proteins, transported to the peroxisomal membrane and translocated into the lumen of peroxisome. In order for the proteins to be transported into the peroxisomal matrix, they must contain peroxisomal targeting signals (PTSs), either at carboxy-terminus (PTS1) or at amino-terminus (PTS2). Over 90% of the verified peroxisomal proteins in yeasts and mammals contain PTS1, which consists of at least the three amino acids of numerous conservative variants such as SKL at the carboxy terminus (9,10). However, 30% of the identified peroxisomal proteins in Arabidopsis thaliana harbor PTS2, which contains a nonapeptide as a canonical consensus sequence (11,12). Newly synthesized PTS1 proteins are bound by the cytosolic receptor PEX5 and transported to the peroxisome, while PTS2 proteins are transferred by PEX7. Whereas Pex5 functions on its own, Pex7 requires co-receptors such as Pex18 and Pex21 in S. cerevisiae (13) or Pex20 in other yeast species (14,15). Exclusively in mammals, two isoforms of PEX5 have been identified (16). The short isoform of PEX5 (PEX5S) binds only PTS1 proteins while the longer isoform PEX5L, which contains an insert of 37 amino acids, interacts additionally with PEX7 in vitro as well as in vivo (16,17).

PEX5 or PEX7, carrying the respective cargo, then attaches to the peroxisomal membrane via a docking complex composed of at least the peroxisomal membrane proteins, PEX13 and PEX14. In yeast, a third component, a peripheral membrane protein Pex17, has been identified and was shown to interact with Pex5 via its direct interaction with Pex14 (18). The N-terminal domain of PEX5 constitutes distinct pentapeptide motifs, WXXX(F/Y), which mediate the interaction with the N-terminal region of PEX14 in mammals (19–21). Whereas PEX7 binds directly to PEX13 and PEX14 in yeast (22,23), it requires PEX5L in mammals, which then associates with the docking complex (24,25).

How the docking of cargo-bound receptors subsequently leads to the translocation of cargo into the peroxisomal matrix is as of yet poorly understood. The RING-finger complex comprising PEX2, PEX10 and PEX12 may contribute to the translocation and release of peroxisomal matrix proteins. In H. polymorpha, a peripheral membrane protein Pex8 at the luminal side of the peroxisome was suggested to assist the dissociation of PTS1 proteins from the receptor (26). Similarly, S. cerevisiae Pex8 is necessary as a physical link between the docking complex and the RING-finger complex (27). Whether this translocation mechanism involving Pex8 function is conserved in humans remains elusive as a homolog or an ortholog of Pex8 has not yet been identified. A recent study in S. cerevisiae suggests that upon association with Pex14, Pex5 undergoes a conformational change, which enables its insertion as an oligomer into the peroxisomal membrane to form a pore and release the cargo into the matrix (28). On the other hand, Pex7 seems to enter the lumen of the peroxisome completely with its cargo (29).

PEX13 is an integral peroxisomal membrane protein containing an Src homology 3 (SH3) domain at the C-terminus, which faces the cytosol (30,31). Yeast two-hybrid screening with the SH3 domain of S. cerevisiae Pex13 identified Pex5 as a major binding partner and the interaction was confirmed in vitro using a ligand blot assay (30). Moreover, the SH3 domain of Pex13 is involved in binding of the canonical PXXP motif within the N-terminal region of Pex14 (22,23). However, in vitro competition assays demonstrated that Pex5 and Pex14 bind to the SH3 domain of Pex13 simultaneously, suggesting that the binding sites for both proteins do not overlap in Pex13 (32). In contrast, mammalian PEX13 was shown to bind PEX5 through its N-terminal domain in GST pull-down assay whereas it interacted with PEX14 via C-terminal region (20).

In a previous study, we identified the W313G missense mutation in PEX13 as the primary cause of a human peroxisome biogenesis defect in a ZSS patient (PBD HR-11; (33)). Interestingly, W313 is the only tryptophan residue found within the SH3 domain of PEX13 and is highly conserved in almost all SH3 domain proteins from yeast to human. Thus, we investigated if and how this mutation would affect PEX13 function and interaction with other peroxisomal proteins involved in the peroxisome biogenesis. Here, we show that the W313G disrupts PTS1 import but not PTS2 import. We also demonstrate that W313G does not abolish PEX13 binding to PEX14. Furthermore, for the first time, we show that PEX13 interacts with itself in vivo, using live cell FRET microscopy. The W313G mutation interferes with PEX13 homooligomerization and displays a dominant-negative effect on wild-type (WT) PEX13 function. Interaction studies with truncation constructs of PEX13 indicate that the N-terminal region is sufficient for localization at the peroxisome, which in turn seems to be required for mediating the self-association of PEX13.

RESULTS
The W313G mutation in PEX13 disrupts import of PTS1 proteins into peroxisomes
In a previous study, we complemented fibroblast cells of ZSS patients with various PEX expression constructs and identified various novel mutations in PEX genes (33). The W313G mutation in PEX13 was of particular interest, since alignments of over 200 SH3 proteins revealed that W313 is the second most conserved residue in the SH3 domain (34). Although absence of the W313G mutation in control alleles suggested that it is a disease-causing mutation (33), its pathogenicity was verified additionally by transfecting the PEX13-deficient fibroblasts.
with the expression constructs containing the W313G mutation. As expected, EGFP containing a strong PTS1 (SKL) as well as catalase, which contains a weak non-canonical PTS1, was localized at the peroxisomes in the mutant fibroblasts transfected with WT PEX13 (Fig. 1A and B). However, both EGFP-SKL and catalase were mostly found in the cytosol in the fibroblasts complemented with the W313G expression construct (Fig. 1C and D). 22.9% of seeded fibroblasts transfected with WT PEX13 showed peroxisomal catalase, whereas only 4.4% of total fibroblasts transfected with PEX13 W313G revealed complementation (Supplementary Material, Fig. S1). Thus, W313G disrupts PEX13 function in the PTS1 protein import. Since PEX13 contributes to the import of PTS2 proteins as well (35), localization of thiolase, a peroxisomal enzyme containing PTS2, was studied in the PEX13-deficient fibroblasts. Interestingly, thiolase could be found in vesicular structures, suggesting

Figure 1. The W313G mutation in PEX13 disrupts PTS1 import. (A–D): The W313G mutant fibroblasts were cotransfected with EGFP–SKL and either WT PEX13 (A and B) or PEX13W313G (C and D). Two days after transfection, fibroblasts were stained with rabbit anti-catalase and subsequently with anti-rabbit-Cy3. WT PEX13 can restore peroxisomal defect in the W313G mutant fibroblasts as revealed by punctate localization of both EGFP–SKL (A) and catalase (B) in the peroxisomes. However, mutant fibroblasts transfected with PEX13W313G show cytosolic distribution of EGFP–SKL (C) and catalase (D). Nuclei are visualized by blue staining of DAPI. Scale bar = 20 μm. (E–G) The W313G mutant fibroblasts were co-labeled with rabbit anti-thiolase (E) and mouse anti-ALDP (F) and subsequently with anti-rabbit-Cy3 (E) and anti-mouse-Alexa Fluor 488 (F). Overlay demonstrates co-localization between thiolase and ALDP at the peroxisomes (G). Nuclei are visualized by blue staining of DAPI. Scale bar = 10 μm. (H and I) The W313G mutant fibroblasts (H) and WT fibroblasts (I) were permeabilized with 25 μg/ml digitonin and subsequently stained with rabbit anti-thiolase and anti-rabbit-Cy3. Cytosolic distribution of anti-thiolase antibodies rather than the punctate pattern demonstrates that thiolase is inaccessible to the antibodies. Nuclei are visualized by blue staining of DAPI. Scale bar = 20 μm.
that the import into the peroxisomal matrix is mostly unaffected (Fig. 1E). To verify that thiolase is indeed localized at the peroxisome, the W313G mutant fibroblasts were co-labeled against ALDP, a peroxisomal membrane protein (Fig. 1F; (36)). Co-localization of thiolase with ALDP (Fig. 1G) demonstrates that thiolase is expressed at the peroxisome. In addition, it was investigated whether thiolase is correctly imported into the peroxisomal matrix in the W313G mutant fibroblasts as in WT cells. When cells are treated with digitonin instead of Triton X-100, only the plasma membrane is permeabilized and subsequently proteins within the peroxisomal matrix are inaccessible to exogenous antibodies (37). Ubiquitous distribution of anti-thiolase antibody in cytosol in both W313G mutant fibroblasts (Fig. 1H) and WT fibroblasts (Fig. 1I) permeabilized with 25 μg/ml digitonin demonstrates that thiolase is correctly imported into the peroxisomal matrix in the W313G mutant fibroblasts. Thus, the W313G mutation seems to specifically impede PEX13 function in PTS1 protein import.

It was previously reported that the I326T mutation found in human PEX13 is a temperature-sensitive allele as peroxisomal import of PTS1 and PTS2 proteins was restored when the mutant cells were incubated at 30°C (38). In order to test whether the W313G mutation is similarly dependent on the temperature, we analyzed peroxisomal import of catalase after incubating the mutant fibroblasts at 30°C for 7 days. Whereas PEX6 deficient fibroblasts, which display temperature-sensitive defect in catalase import, revealed an increased punctate pattern of catalase, cytosolic distribution of catalase in the W313G cells persisted at 30°C (Supplementary Material, Fig. S2). In I326T patient cells, PEX13 localization at the peroxisome was impaired (36). Similarly, PEX14 was also missing at the peroxisome in I326T mutant cells, although PEX14 amount detected in whole cell lysates was equivalent to PEX14 expression in WT cells. Thus, localization of endogenous PEX14 at the peroxisome seems to be dependent on peroxisomal expression of PEX13. We analyzed whether the W313G mutation also affects the peroxisomal targeting of PEX13 and subsequently the localization of PEX14 at the peroxisome. In contrast to I326T mutant cells, PEX13 is localized in a punctate pattern in the W313G patient fibroblasts (Fig. 2A). Double labeling with peroxisomal ALDP (Fig. 2B) reveals co-localization with PEX13 (Fig. 2C), verifying that PEX13 is correctly localized at the peroxisome. Next, we double labeled the W313G fibroblasts for PEX13 and PEX14 to check whether PEX14 is also expressed at the peroxisome. As expected, PEX14 is found in the punctate pattern (Fig. 2E). Co-localization with PEX13 verifies that PEX14 is also correctly localized at the peroxisomes in the W313G mutant fibroblasts (Fig. 2F). Moreover, the amount of PEX13 and PEX14 in whole cell lysates of the W313G fibroblasts was comparable with the expression in WT cells (Fig. 2G). These findings suggest that the peroxisomal defect in the W313G fibroblasts is not the consequence of mislocalization or altered expression of PEX13 or PEX14. Although I326T and the W313G are both missense mutations located within a close proximity of the SH3 domain of PEX13 and both impede PTS1 import, the underlying mechanisms of their pathogenicity appear to be different.

While analyzing PEX14 localization in the W313G fibroblasts, we observed that the peroxisomes were less abundant in the patient cells than in WT cells (Fig. 3A and B). In order to quantify this observation, the peroxisomes were labeled with an anti-PEX14 antibody and counted using ImageJ software. To compensate for large variations in cell sizes, the total number of peroxisomes in each cell was divided by the cell area. Whereas the W313G fibroblasts contained 7.5 ± 1.5 peroxisomes/100 μm² on average, WT cells showed 15.6 ± 3.7 peroxisomes/100 μm², confirming the microscopic observation (Fig. 3C). Thus, the W313G mutation appears to affect the peroxisome abundance. Similarly, an earlier study reported that ZSS patient fibroblasts with mutations in PEX1, PEX2, PEX5, PEX6 and PEX10 also revealed reduced abundance of peroxisomes (39). It was postulated that the peroxisome abundance may be dependent on the metabolic state as defect in peroxisomal β-oxidation also led to a reduction in the peroxisome numbers. However, the mechanism underlying the peroxisome abundance in ZSS patients is yet unclear.

The W313G mutation does not abolish PEX13 interaction with PEX14

Numerous studies in yeast and mammalian cells have demonstrated that the SH3 domain of PEX13 binds to PEX14 (30,31,40,41). A previous study in S. cerevisiae revealed that substitution of W349 with alanine abolishes Pex13 interaction with Pex14 specifically (32). Interestingly, W313 in human PEX13 corresponds to W349 in S. cerevisiae (33). Thus, we tested whether the W313G mutation would disrupt PEX13 binding to PEX14 using co-immunoprecipitation. It was first tested whether the c-Myc epitope tag at the carboxy end of PEX13 would interfere with the PEX13 function since it was found in S. cerevisiae that the hemagglutinin tag at the carboxy terminal end of Pex13 could not complement Δpex13 strain functionally, indicating that an epitope tag at the C-terminus disturbs Pex13 function (40). However, catalase was found in vesicular structures, indicative of the peroxisomes, in the W313G mutant fibroblasts complemented with PEX13-Myc, demonstrating that PEX13-Myc is functional (Fig. 4A and B). Notably, 13.4% of transfected cells showed PEX13-Myc and 13.7% of cells revealed peroxisomal catalase (Supplementary Material, Fig. S3). Thus, peroxisomal import of catalase appears to be restored in nearly all cells expressing PEX13-Myc. Interestingly, the transfection rate of W313G-Myc (7.9%) was lower compared with PEX13-Myc. Whole cell lysates from HeLa cells transfected with PEX13-Myc and pcDNA3.1-PEX14 were first immunoprecipitated with an anti-c-Myc antibody and then detected using an anti-PEX14 antibody. In contrast to W349A substitution in S. cerevisiae, the corresponding W313G mutation does not abolish PEX13 interaction with PEX14 completely (Fig. 4C). In order to exclude the possibility that PEX14 may bind unspecifically to the c-Myc epitope, YB1-Myc (Y box binding protein 1), which is localized at the nucleus, was analyzed as a control. A lack of PEX14 interaction with YB1, despite much stronger expression and immunoprecipitation with anti-c-Myc, further demonstrates that PEX14 binding to PEX13 or W313G is specific. In consistence with band intensities of anti-PEX14 shown in the western blot analysis, the relative density of anti-PEX14 bands in co-immunoprecipitation with W313G-Myc is slightly less than with WT PEX13-Myc, 0.43 ± 0.08 and 0.57 ± 0.08, respectively (Fig. 4D). However, it should be noted that the relative density of anti-c-Myc shows comparable
reduction: $0.41 \pm 0.05$ for W313G-Myc and $0.59 \pm 0.05$ for PEX13-Myc. In addition, the transfection rate of W313G-Myc was also less than PEX13-Myc (Supplementary Material, Fig. S3). Thus, the weaker band intensity of anti-PEX14 in co-immunoprecipitation with W313G-Myc is more likely to be the consequence of less W313G-Myc protein immunoprecipitated rather than an indication of significantly reduced interaction between W313G-Myc and PEX14.

Yeast two-hybrid screening with the SH3 domain of *S. cerevisiae* Pex13 identified Pex5 as a major binding partner, and the interaction was confirmed in vitro using a ligand blot assay (30). The SH3 domain of PEX13 was also shown to bind PEX5S and PEX5L through a yeast two-hybrid assay (31). Furthermore, mammalian PEX5 could be recovered with GST-PEX13 in GST pull-down assay (20). Thus, we investigated whether the W313G mutation would affect PEX13 binding to PEX5 using co-immunoprecipitation. Despite numerous attempts using diverse buffer conditions, PEX5 could not be detected in immunoprecipitates of PEX13-Myc. Although PEX14 could be readily co-purified using PEX13-Myc, the c-Myc epitope at the carboxy terminal end may disturb PEX13 interaction with PEX5 specifically. However, co-immunoprecipitates could not be detected even when untagged PEX5 and PEX13 were transfected. It should be noted that similar failure in detection of PEX13 binding to PEX5 has been reported for human PEX13 (42) as well as in *Arabidopsis* (43).

PEX13 interacts with itself in live cells

To investigate PEX13 interaction directly in live cells, we applied spectrally resolved FRET measurements, which allow for the measurement of apparent FRET efficiency as well as the relative abundances of donor and acceptor (44,45). First, we generated PEX13 and PEX14 constructs fused to Citrine or Cerulean in EGFP-N1 vector backbone. As expected, PEX13-Citrine or -Cerulean was correctly targeted to the peroxisome (Fig. 5A). In contrast, PEX14-Citrine was mislocalized to the tubular structures, reminiscent of mitochondria (Fig. 5B). Since it was observed in *S. cerevisiae* that simultaneous overexpression of both Pex13 and Pex14 rescues growth defect of either
To substantiate this unexpected finding, we investigated whether PEX13 can be co-immunoprecipitated with itself. HeLa cells were cotransfected with PEX13-Myc and PEX13-mGFP to differentiate tagged PEX13 from endogenous PEX13 in the western blot. However, we could not detect PEX13-mGFP using 0.1% digitonin in the co-immunoprecipitation buffer. Agne et al. reported previously that only 1% digitonin showed to be suitable for dissociation of sufficient amounts of intact protein complexes from the peroxisomal membrane (27).

Thus, we investigated whether PEX13–PEX13 interaction could be isolated using 1% digitonin in the buffer. Indeed, PEX13-mGFP could be clearly detected after immunoprecipitation with PEX13-Myc (Fig. 6A). Quantification of relative density (Fig. 6B) revealed that the relative density of anti-c-Myc bands was again lower for W313G-Myc (0.47 ± 0.04) than for PEX13-Myc (0.53 ± 0.04). Whereas co-immunoprecipitation with PEX13-Myc showed 0.71 ± 0.13 relative density for anti-GFP bands, co-immunoprecipitation with W313G-Myc revealed a lower relative density of 0.29 ± 0.13 (Fig. 6B). This significant decrease in co-immunoprecipitation between W313G-Myc and W313G-mGFP indicates that the W313G mutation interferes with PEX13 interaction with itself. It should be noted that whereas FRET efficiency is measured in living cells with intact peroxisomal membrane, where PEX13 is expressed in its native structure, co-immunoprecipitation includes denaturing conditions. Consequently, PEX13 structure would not be in its native state. Retention of residual interaction between W313G-Myc and W313G-mGFP in the western blot compared with the FRET measurement may likely reflect such difference in the structure of W313G PEX13.

Next, the stability of PEX13 self-association was investigated by immunoprecipitation of whole cell lysates at various temperatures. While PEX13-mGFP can be still co-isolated with PEX13-Myc even at a high temperature of 80°C, W313G-mGFP is barely detectable (Fig. 6C). Detection with an anti-c-Myc antibody verified that the amount of immunoprecipitated PEX13 is comparable at all three temperatures. This reduced stability of PEX13 proteins containing the W313G mutation suggests that the W313 residue plays an important role in maintaining the PEX13 oligomer.

We then asked whether endogenous PEX13 function would be affected when the W313G mutant PEX13 is simultaneously expressed. HeLa cells transfected with either WT or the W313G mutant PEX13 tagged to mGFP were stained with an anti-catalase antibody and counted for cytosolic localization of catalase. Interestingly, catalase was mislocalized to the cytosol in 26.6% of HeLa cells containing the W313G mutant PEX13, whereas only 2.9% of cells carrying WT PEX13 showed catalase. Interestingly, catalase was mislocalized to the cytosol in 26.6% of HeLa cells containing the W313G mutant PEX13, whereas only 2.9% of cells carrying WT PEX13 showed catalase (Fig. 6D). Thus, the W313G mutation appears to have a dominant-negative effect on WT PEX13 function in at least PTS1 import.

N-terminal domain is essential for PEX13 oligomerization

In S. cerevisiae, yeast two-hybrid screening with the SH3 domain of Pex13 identified Pex5 as a major binding partner (30). In vitro binding assays using the SH3 domain of Pex13 fused to GST demonstrated that the SH3 domain alone is responsible for the interaction with Pex5 in Pichia pastoris (41). In contrast, mammalian PEX13 was shown to bind PEX5 through its
N-terminal domain as PEX5 was recovered in the GST pull-down assay with GST-PEX13N, containing only the N-terminal region, but not with GST-PEX13C (20). Since the N- and C-terminal parts of PEX13 clearly play different roles for PEX13 function, we investigated whether the N- or C-terminal or rather both domains would contribute to the oligomerization of PEX13. We generated deletion constructs containing either only N- or C-terminal domains or the SH3 domain, labeled as PEX13N, PEX13C or PEX13 SH3, respectively (Fig. 7A). The PEX13 deletion construct lacking the C-terminal domain was correctly targeted to the peroxisomes, revealed by the typical punctate pattern (Fig. 7B). In contrast, the PEX13C construct with N-terminal domain truncation was mislocalized largely to the tubular structures, reminiscent of mitochondria (Fig. 7C). Double labeling of cells transfected with PEX13C construct with Mitotracker, which visualize mitochondria in living cells, verified that PEX13C is indeed localized mostly in mitochondria (Supplementary Material, Fig. S4A–C). As expected, PEX13 SH3, lacking the transmembrane region, was ubiquitously distributed in the cytosol (Fig. 7D).

C-terminal parts of PEX13 clearly play different roles for PEX13 function, we investigated whether the N- or C-terminal or rather both domains would contribute to the oligomerization of PEX13. We generated deletion constructs containing either only N- or C-terminal domains or the SH3 domain, labeled as PEX13N, PEX13C or PEX13 SH3, respectively (Fig. 7A). The PEX13 deletion construct lacking the C-terminal domain was correctly targeted to the peroxisomes, revealed by the typical punctate pattern (Fig. 7B). In contrast, the PEX13C construct with N-terminal domain truncation was mislocalized largely to the tubular structures, reminiscent of mitochondria (Fig. 7C). Double labeling of cells transfected with PEX13C construct with Mitotracker, which visualize mitochondria in living cells, verified that PEX13C is indeed localized mostly in mitochondria (Supplementary Material, Fig. S4A–C). As expected, PEX13 SH3, lacking the transmembrane region, was ubiquitously distributed in the cytosol (Fig. 7D). After verification of expression in HeLa cells, the FRET efficiency between Citrine- and Cerulean-tagged PEX13 deletion constructs was measured in living cells. Strikingly, PEX13N, which was localized to the peroxisomes, was found to have a FRET efficiency of 0.187 ± 0.021 (n = 11). The W313G mutation significantly decreased the FRET efficiency to 0.118 ± 0.015 (n = 10; P < 0.05), a level similar to that measured in the peroxisome localized negative control, PEX2, which shows a FRET efficiency of 0.125 ± 0.010 (n = 20).
peroxisomes, demonstrated FRET efficiency comparable with the WT PEX13 (Fig. 7E). In contrast, PEX13C and PEX13 SH3 constructs, which were localized outside of the peroxisomes, revealed very low FRET efficiency. This observation suggests that peroxisomal localization of PEX13 is necessary for the oligomerization and that N-terminal half is sufficient.

In addition, we verified whether the deletion constructs would be able to complement the defect in the function of PEX13 carrying the W313G mutation. PEX13N, the only deletion construct found at the peroxisome, could restore peroxisomal targeting of catalase in the W313G mutant fibroblasts (Fig. 8A and B). Moreover, the complementation rate of PEX13N-Citrine (14.8%) was only slightly lower than that...
of PEX13-Citrine (17.9%; Supplementary Material, Fig. S5). In contrast, catalase was found in cytosol of the mutant cells carrying PEX13C or SH3 (Fig. 8C–F). This result implies that PEX13 does not require the C-terminal domain, including the SH3 domain for restoration of PTS1 import in the W313G mutant fibroblasts.

**DISCUSSION**

Here, we investigated the molecular and biochemical mechanisms of peroxisomal defects induced by the W313G mutation in human PEX13. Whereas import of PTS1 proteins was severely impeded, PTS2 protein import was mostly undisturbed. This observation was unexpected since PEX13 is well accepted as a point of convergence for both PTS1 and PTS2 protein import. Moreover, all previously described mutations identified in human PEX13 were shown to abolish PTS1 as well as PTS2 import (38,47).

Remarkably, interaction studies using FRET microscopy techniques in live cells revealed that PEX13 interacts with itself. In addition, PEX13 could be co-isolated from whole cell lysates using PEX13-Myc. In earlier work, PEX13 was isolated as a large protein complex with very high molecular mass when rat liver peroxisomes were solubilized using digitonin (48). Contrary to the general assumption that mammalian PEX13 interacts...
with PEX14 and PEX5, the majority of PEX13 was not associated with PEX5 or PEX14, suggesting strongly that PEX13 is part of a complex distinct from the PEX14-PEX5 containing complex. Similarly, direct interaction between PEX5 and PEX13 could not be detected despite various attempts (42,49). It should be noted that we could not detect binding of PEX13 to PEX5 using co-immunoprecipitation, either. Further purification of the PEX13-containing complex and analysis through MALDI-MS revealed PEX13 as a major, if not the only, component (48). This striking observation led to the postulation that PEX13 is part of a complex distinct from the PEX14-PEX5 containing complex. Similarly, direct interaction between PEX5 and PEX13 could not be detected despite various attempts (42,49).

It should be noted that we could not detect binding of PEX13 to PEX5 using co-immunoprecipitation, either. Further purification of the PEX13-containing complex and analysis through MALDI-MS revealed PEX13 as a major, if not the only, component (48). This striking observation led to the postulation that PEX13 may not be restricted to mammalian PEX13. Although the W313G patient fibroblasts were transfected with PEX13N-Citrine and subsequently stained with a rabbit anti-catalase antibody as above. Cells carrying PEX13N-Citrine and subsequently stained with a rabbit anti-catalase antibody. Cells carrying PEX13N-Citrine and subsequently stained with a rabbit anti-catalase antibody. Cells carrying PEX13N-Citrine and subsequently stained with a rabbit anti-catalase antibody as above. Cells carrying PEX13NCitrine (A) reveal partially restored import of catalase in the peroxisomes (B). (C–F) Catalase distribution was investigated in the W313G patient fibroblasts transfected with PEX13C-Citrine (C and D) or PEX13 SH3-Citrine (E and F) and subsequently stained with a rabbit anti-catalase antibody as above. Cells carrying PEX13C-Citrine (C and D) or PEX13 SH3-Citrine (E and F) show ubiquitous distribution of catalase in the cytosol (D and F). Scale bar = 10 μm.

Figure 8. N-terminal domain of PEX13 can partially restore PTS1 import defect in the W313G fibroblasts. (A and B) The W313G mutant fibroblasts were transfected with PEX13N-Citrine and subsequently stained with a rabbit anti-catalase antibody. Cells carrying PEX13N-Citrine (A) reveal partially restored import of catalase in the peroxisomes (B). (C–F) Catalase distribution was investigated in the W313G mutant fibroblasts transfected with PEX13C-Citrine (C and D) or PEX13 SH3-Citrine (E and F) and subsequently stained with a rabbit anti-catalase antibody as above. Cells carrying PEX13C-Citrine (C and D) or PEX13 SH3-Citrine (E and F) show ubiquitous distribution of catalase in the cytosol (D and F). Scale bar = 10 μm.

In order to gain more insight into why the W313G mutation within the SH3 domain would attenuate oligomerization of PEX13, we analyzed the molecular characteristics and structure of PEX13 induced by the W313G substitution using web services such as SNPeffect (http://snpeffect.switchlab.org; (53)) and HOPE (http://www.cmbi.ru.nl/hope/; (54)). Interestingly, SNPeffect describes that the W313G mutation decreases the aggregation/oligomerization tendency of PEX13 (Fig. 9A) using a statistical mechanics algorithm, TANGO, which predicts the aggregation prone regions in a protein sequence (55). Consistent with our observation that the N-terminal and transmembrane domain alone can mediate sufficient oligomerization of PEX13, amino acid residues showing the highest TANGO aggregation score are located within the N-terminal and transmembrane domain (Fig. 9A and Table 1). Moreover, HOPE predicts that substitution of tryptophan, which contains an indole functional group and is the largest amino acid residue, through much smaller glycine may lead to the loss of hydrophobic interactions with other proteins (Fig. 9B).

Together, our data suggest that (i) homooligomerization of PEX13 is essential for PEX13 function in the import of PTS1 protein but not of PTS2 protein; (ii) homooligomerization is not important for PEX13 interaction with PEX14 and (iii) the N-terminal domain alone is sufficient for peroxisomal localization of PEX13 and homooligomerization.
MATERIALS AND METHODS

Cell culture
WT or patient fibroblast cells containing the W313G mutation as well as HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (low glucose) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin and 100 U/ml streptomycin at 37°C with 5% CO2.

Molecular cloning
pcDNA3.1-PEX13W313G was generated using a QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) with pcDNA3.1-PEX13 (33) as template. To clone PEX13-Myc or PEX13W313G-Myc, PEX13 or PEX13W313G ORF was amplified with 5′XbaI and 3′Hind III site and ligated into pcDNA3.1myc/-His(-)A (Invitrogen). PEX13-Citrine and -Cerulean was generated by cloning PEX13 ORF into pCitrine or pCerulean vectors, which contain either Citrine or Cerulean in place of EGFP in EGFP-N1 (Clontech) vector backbone, via 5′XhoI site and 3′Hind III site. The same sites were used to clone truncation constructs of PEX13 (PEX13N, PEX13C and PEX13 SH3) for FRET experiments. For PEX14-Citrine and -Cerulean, 5′HindIII and 3′SacII sites were used. For PEX2-Citrine and -Cerulean, 5′XhoI and 3′SacII sites were used. PEX13-mGFP was cloned via 5′BamHI and 3′NotI sites in pcDNA3-mGFP vector.

Antibodies
Following primary antibodies were used: rabbit anti-catalase (Oxis International Inc.), goat anti-PEX13 (Abcam), rabbit anti-PEX14 (ProteinTech Group), rabbit anti-thiolase (ACAA1; ProteinTech Group), mouse anti-ALDP (Euromedex), mouse anti-GAPDH (Abcam) and mouse anti-c-Myc (Cell Signaling Technology, Inc.). Species-specific secondary antibodies conjugated to Cy3 (Jackson ImmunoResearch Laboratories, Inc.), Alexa Flur 488 (Invitrogen) or horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc.) were used.

Fluorescence microscopy and image analysis
Cells were grown on coverslips (Menzel-Gläser; Thermo Fisher Scientific) in 12-well plates and transfected using Effectene (Qiagen) and 0.3 mg of total DNA per well. After 48 h, cell were fixed in 4% (w/v) paraformaldehyde in Phosphate buffered saline (PBS) for 20 min and permeabilized with 1% (v/v) Triton X-100 in PBS for 5 min. Subsequently, the cells were incubated at room temperature for at least 1 h with the primary and secondary antibodies. Finally, cells were mounted in ProLong Gold containing DAPI (Invitrogen) to label the nuclei. Images were acquired with an epifluorescence microscope (Axio Imager M1; Carl Zeiss) and AxioCam HRm camera (Carl Zeiss) using AxioVision software (Carl Zeiss). For oil immersion, Immersol 518F (Carl Zeiss) was used. AxioVision or Photoshop CS3 software was used to adjust the brightness or contrast. ImageJ software (National Institutes of Health) was applied to count the peroxisomes and to measure the cell area in single images. At least three experiments were quantified comprising >20 cells in total for each cell line analyzed.

Immunoprecipitation
HeLa cells were seeded at 106 in a 10 cm plate and transfected with Myc constructs using Effectene (Qiagen). 48 h after

Figure 9. Prediction of structural disturbance through the W313G substitution. (A) Bar representation of the TANGO windows present in the WT and the W313G mutant protein (W313G). Schematic drawing of PEX13 is shown on the top as in Fig. 6A. In the bar representation, the position of the aggregating stretches is visualized in red. The dashed vertical line indicates loss of aggregation propensity at the position of the W313G mutation. (B) Close-up model of the W313G substitution in PEX13. Tryptophan residue is shown in green, while the rest of protein is represented in gray.
transfection, cells were collected and resuspended in Co-IP buffer [20 mM Tris, pH 7.4; 0.15 M NaCl; 1% digitonin; protease inhibitor (Complete Cocktail Tablet, Roche)]. After homogenization, cell extracts were centrifuged at 20 000 g for 20 min and the supernatant containing protein lysates was collected. Protein concentration was measured through a bicinchoninic acid assay (Upima). 5 × Sodium dodecyl sulphate (SDS) sample buffer was added to 10 µg of protein lysate as an input control. Two hundred and fifty micrograms of total protein lysates was incubated with antibodies overnight at 4 °C. Next day, 20 µl Protein A/G Plusagarose (Santa Cruz Biotechnology, Inc.) was added and incubated with protein lysates containing antibody for additional 3 h at 4 °C. Then, the beads were centrifuged at 2500 g and the supernatant was discarded. After washing the beads three times with 1 ml PBS as described by the manufacturer, proteins were eluted in 20 µl SDS sample buffer and analyzed by the western blot. For temperature test, a signal was detected using the ECL reagent (Lumi-Light; Roche) by LAS-4000 mini (Fujifilm). Band intensity of the western blot was measured using Multi Gauge software (Fujifilm).

**Live cell spectral imaging**

For imaging of live cells, cover slips were placed in custom-made image acquisition chambers with 500 µl of PBS. Images were acquired on a Zeiss LSM 510-Meta confocal microscope (Carl Zeiss) equipped with a 40 × oil-immersion objective (NA 1.3). For direct excitation of Cerulean, the 458 nm laser line was used and emission collected over eight channels spanning 464 – 636 nm. For direct excitation of Citrine, the 488 nm laser line with the emission channels shifted to detect 497 – 583 nm was used. All emission was collected through a 170 µm pinhole and images were digitized/collection with 12-bit resolution.

**Spectral FRET analysis**

In order to minimize artifacts resulting from the peroxisome movement during acquisition and/or misalignment of dichroic mirrors, per pixel FRET analysis was not performed. Rather, background subtracted total image intensity was computed and used. In each FRET measurement, non-negative linear unmixing of the background corrected FRET sample spectra was performed using background subtracted reference spectra. The reference spectra were measured from images of cells expressing selectively donors (PEX13-Cerulean) or acceptors (PEX13-Citrine). These images were acquired with the same excitation and detection parameters as used for the FRET image acquisition. Calculation of apparent FRET efficiencies from the unmixed apparent concentrations has been described elsewhere (44). In addition to apparent FRET efficiencies from the unmixed apparent concentrations has been described elsewhere (44). Only measurements with equal amounts of donors and acceptors (donor mole fraction equal to 0.5 ± 0.1) were used in the comparison of apparent FRET efficiency. The above analysis was performed using Matlab 7.6.0 (2008a; The MathWorks).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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