Drosophila models of peroxisomal biogenesis disorder: peroxins are required for spermatogenesis and very-long-chain fatty acid metabolism

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Peroxisomes are vital eukaryotic organelles that participate in lipid metabolism, in particular the metabolism of very-long-chain fatty acids (VLCFA). The biogenesis of peroxisomes is regulated by a set of peroxin proteins (PEX). In humans, mutations affecting peroxin protein production or function result in devastating diseases classified as peroxisome biogenesis disorders (PBD). The way in which peroxisomal dysfunction leads to the pathophysiological consequences of PBD is not well understood. Here we report that Drosophila pex mutants faithfully recapitulate several key features of human PBD, including impaired peroxisomal protein import, elevated VLCFA levels and growth retardation. Moreover, disruption of pex function results in spermatogenesis defects, including spermatocyte cytokinesis failure in Drosophila. Importantly, increased VLCFA levels enhance these spermatogenesis defects whereas reduced VLCFA levels alleviate them. Thus, regulation of proper VLCFA levels by pex genes is crucial for spermatogenesis. Together our study reveals an indispensable function of pex genes during spermatogenesis and provides a causative link between the phenotypic severity of pex mutants and VLCFA levels.

INTRODUCTION

Peroxisomes are single membrane bound organelles present in almost all eukaryotic cells. They are critical for the metabolism of fatty acids and many other metabolites. The amorphous peroxisomal matrix hosts more than 50 enzymes that participate in various metabolic reactions (1,2). The importance of peroxisome function is underscored by the existence of a class of devastating human diseases named peroxisomal biogenesis disorders (PBD) in which import of the peroxisomal matrix or membrane proteins is impaired (3,4). Occurring at a frequency of 1/25 000 to 1/50 000 births, PBD is characterized by metabolic homeostatic defects as well as developmental abnormalities including facial malformations, and growth and mental retardation. PBD often leads to death in infancy or childhood and currently there is no effective treatment or cure.

In yeast and plants, degradation of fatty acids by β-oxidation is carried out exclusively in peroxisomes. In animals, β-oxidation of fatty acids with carbon chains shorter than 20 mainly occurs in mitochondria, although β-oxidation of very-long-chain fatty acid (VLCFA, C20 and up) occurs exclusively in peroxisomes (1). Therefore, elevated VLCFA levels, in particular of C24 and C26 VLCFAs, is a hallmark of PBD (4,5). Since peroxisomes also participate in other metabolic pathways such as H2O2 production and decomposition, ether lipid biosynthesis and glyoxylate metabolism, the contribution of elevated VLCFAs to PBD pathogenesis remains uncertain (4,6,7). Nevertheless, therapeutic treatments aiming to lower the VLCFA level have been developed for PBD and other VLCFA accumulation diseases, such as adrenoleukodystrophy (ALD), which is caused by mutations in the putative fatty acid transporter ABCD1. For example, in combination with a low VLCFA diet, Lorenzo’s
oil, a specific mixture of oils that may inhibit fatty acid biosynthesis, has been used to treat PBD, although with limited success (7).

All peroxisomal proteins are synthesized in the cytoplasm and imported into peroxisomes using peroxisome targeting signals (PTS). Protein components required for peroxisome biogenesis are collectively called peroxins. At least 12 peroxin genes (PEX genes) have been linked to PBD. Among these, PEX1, 2, 5, 6, 7, 10, 12, 13 and 14 are required for PTS1- or PTS2-containing peroxisomal matrix protein import, although PEX3, 16 and 19 are essential for peroxisomal membrane protein targeting (8–10). Three ring finger domain-containing peroxisome membrane proteins, PEX2, PEX10 and PEX12, form a complex, although the exact molecular function of this complex is unknown (11,12). The disease-related peroxins are highly conserved throughout evolution, with orthologs present in yeast, worm, flies, mice and humans. In addition to the conserved peroxins, species-specific peroxins have also been identified in yeast and humans (9,13).

To gain more insight into PBD pathogenic mechanisms and to establish experimental systems for treatment development, a number of PBD models have been generated. Like PBD patients, pex2, pex5 and pex13 knockout mice exhibit growth retardation, increased VLCFA levels and neonatal lethality (14–16). How peroxin disruption leads to these phenotypes is not clear. In Caenorhabditis elegans, RNAi of the pex5 homolog, prx-5, led to postembryonic L1 stage arrest (17). In Arabidopsis, mutants of PEX2, PEX10 and PEX12 are all embryonic lethal, indicating that peroxisomes are essential for plant development (18).

The Drosophila genome contains 15 peroxin genes. Their functions are largely unexplored. Here we show that pex genes are important for spermatocyte development. Increasing the amount of VLCFA but not long-chain fatty acid (LCFA) in the diet significantly enhances the spermatocyte developmental defects of pex mutants. In addition, pex genetically interacts with bond and Sc2, two VLCFA biosynthesis genes, further supporting the conclusion that VLCFA homeostasis regulated by pex genes is crucial for spermatogenesis in Drosophila. The genetic properties of pex mutants afford an opportunity for discovering pathways and processes that interact with VLCFA metabolism, which may subsequently facilitate the development of intervention strategies for PBD.

RESULTS

Drosophila PEX2 and PEX10 are essential for male fertility

We identified 15 Drosophila peroxin genes by performing Blast searches using the protein sequences of all known peroxins from yeast and humans (Supplementary Material, Table S1). Except PEX1 and PEX6, all the encoded peroxins share ~30% identity and ~50% similarity to their human counterparts throughout the whole protein coding region (Supplementary Material, Table S1). Although many mutants are available for these pex genes, little information regarding their mutant phenotypes and potential function during Drosophila development has been reported. Thus, as a first step in determining pex gene function in development, we examined the mutant phenotypes of several pex genes, in particular the ring finger protein-coding genes pex2, pex10 and pex12.

The pex2(y01889) P-element insertion mutation is homozygous lethal, but in trans to a deficiency (DfExel6112) is viable with sterility in males and reduced fertility in females (Fig. 1A and data not shown). We could separate the lethality from the male sterility through recombination, indicating that the lethality is due to a mutation in another gene. A second P-element insertion allele, pex2(HP35059), is homozygous viable and male sterile (Fig. 1A). The male sterility in pex2 mutants could be fully rescued by a pex2 transgene (see below), indicating that pex2 is not essential for viability but is required for male fertility. Next, we asked how the pex2 mutant leads to male sterility.

In wild-type testis, round spermatocytes and elongated spermatid bundles can be easily identified (Fig. 1C). In contrast, pex2 mutant testes contain many spermatocytes and some ‘cotton ball’-like spheres but completely lack elongated spermatid bundles and mature sperm (Fig. 1C). Therefore pex2 may specifically affect male germ cell development.

To address whether other peroxin genes play roles in male fertility, we generated pex10 deletion mutants through P-element imprecise excision. Both pex10 alleles obtained were putative null alleles based on their molecular lesions and their phenotypes when in trans with a deficiency (Fig. 1A and B). Not surprisingly, pex10 mutants are viable with reduced female fertility and are male sterile with the same testis phenotype as pex2 mutants (Fig. 1C). We also obtained a pex12(y01360) P-element insertion mutant and found a similar testis phenotype (data not shown). These data are consistent with previous findings that PEX2, PEX10 and PEX12 function in a complex (12). Moreover, the testis phenotypes in pex2 mutants progressively worsened with age. The mutant testes appear much thinner and longer on the seventh day after eclosion than they did immediately after eclosion (Fig. 1C). In addition to the male sterile phenotype, the pex2 mutants all display growth defects: mutants have a 2-day delay in eclosion (data not shown). We conclude that PEX2, PEX10 and PEX12 are essential for Drosophila male fertility.

PEX2 and PEX10 are required for spermatocyte growth and cytokinesis

Next, we examined the male sterile phenotype in detail with phase-contrast microscopy and with DNA staining in both pex2 and pex10 mutants. Before the spermatogonial stage, pex mutant testes are indistinguishable from wild-type. After the spermatogonial stage, wild-type spermatocytes go through the G1/S phase and DNA replication, followed by a 3.5-day spermatocyte growth phase (G2 phase). At this stage, primary spermatocytes are characterized by large nuclei, prominent nucleoli and three masses of chromosomal DNA. Once the primary spermatocytes mature, the nucleoli disappear and the chromosomes start condensing, forming three compact dots before the meiotic divisions begin (G2/M phase) (Fig. 2A–C) (19). In pex mutant testes, growing primary spermatocytes could be identified easily, but hardly any normal mature spermatocytes were detected. Instead, only a few fused spermatocytes with condensed chromosomes were found (Fig. 2F–H and K–M). We also quantified the number of spermatocytes per testis and found that there are...
more spermatocytes in \textit{pex} mutants than wild-type (\textit{pex2}: 426 ± 88; \textit{wt}: 317 ± 81; Student’s \textit{T}-test \( P < 0.001; N = 15 \)). To rule out the possibility that the fused spermatocytes were the consequence of excessive squashing of the testis samples, we examined intact testes that were not cut open. \textit{Drosophila} testes contain cysts, each of which harbors 16 developing spermatocytes. Within a cyst, wild-type spermatocytes have clear cell–cell boundaries and do not have rounded nuclei (Fig. 2P). However, in \textit{pex} mutants, cysts of 16 spermatocytes without cell–cell boundaries and with large round nuclei were present, indicating that the fused spermatocytes exist \textit{in vivo} (Fig. 2Q). These results suggest that the spermatocyte growth stage is defective in \textit{pex} mutants. The majority of spermatocytes did not mature, and some were fused. The fused spermatocytes in \textit{pex2} and \textit{pex10} mutants were able to continue developing but exhibited profound defects in spermatid differentiation. In wild-type, each spermatocyte undergoes two rounds of meiotic divisions to form four spermatids. During the onion stage, each round spermatid has one phase bright nucleus and one phase dark mitochondrial derivative (Fig. 2D and R) (19). In both \textit{pex2} and \textit{pex10} homozygous mutants, only a few round spermatids were found compared with wild-type (\textit{pex2}: 38 ± 20; \textit{wt}: 169 ± 40; Student’s \textit{T}-test \( P < 0.001; N = 15 \)) and they often exhibited four or more nuclei associated with a single enlarged mitochondrial derivative (Fig. 2I, N and S). These observations indicate a failure of cytokinesis during meiotic division. Chromosome segregation appears unaffected (data not shown). Moreover, unlike wild-type testes, which have very long spermatid bundles (Fig. 2E), the few spermatids in \textit{pex} mutants, presumably from a cyst, form cotton ball-like spheres (Fig. 2J and O). Thus \textit{pex} mutant spermatids can initiate the extension of sperm tails, but this process is abnormal.

\textbf{pex} Mutants are defective at the spermatocyte G2 growth phase

The appearance of more spermatocytes in \textit{pex} mutants partially resembled the phenotype of mutants of the well-studied spermatocyte arrest genes, which control spermatocyte meiotic entry and differentiation (20). We asked whether the
expression of spermatocyte-arrest genes and a panel of differentiation-related genes are altered in **pex** mutants. Using semi-quantitative RT–PCR we did not detect significant differences in transcript levels, except for the **don juan** gene (Supplementary Material, Fig. S1). **don juan** is a differentiation marker for elongated spermatid bundles (21). RNA **in situ** hybridization with a **don juan** antisense probe confirmed the RT–PCR result. In wild-type, **don juan** is weakly
expressed in early spermatocytes and strongly expressed in late spermatocytes and persists in elongating stages. In *pex* mutants, low levels of *don juan* transcripts were found throughout most of the testis (Supplementary Material, Fig. S1), suggesting that *pex* mutant testes contain mainly early rather than late spermatocytes. Additionally, the expression levels of *pex2* and *pex10* were unchanged in several spermatocyte arrest mutants including *aly* and *can* (Supplementary Material, Fig. S1). Thus the spermatocyte phenotypes in *pex* mutants are probably not due to defects associated with spermatocyte-arrest genes.

To further pin down the defective stage of spermatogenesis in *pex* mutants, we asked whether *pex* mutants have defects in G1/S phase or G2 phase during spermatocyte development. During G1/S phase, replicating DNA can be labeled with BrdU. In *pex* mutants, BrdU staining appeared indistinguishable from wild-type, implying that *pex* mutant spermatocytes go through G1/S phase normally (Fig. 3A and B). We then examined the state of mutant spermatocytes with Cyclin A staining. Cyclin A accumulates in the cytoplasm when spermatocytes enter the G2/M transition (arrow in C). The majority of *pex10* mutants have only cytosolic Cyclin A (arrow in D). A few scattered spermatocytes have nuclear Cyclin A (arrow in E). Scale bar: 100 μm. (F and G) Boule immunostaining. Boule is expressed in many developing wild-type spermatocytes (arrow and enlarged view in F), although in *pex10* mutants very few spermatocytes express Boule (arrow and enlarged view in G). Arrowheads in F and G are non-specific staining of Boule antibodies in spermatid bundles. (H–K) X-Gal staining of a *Twine-lacZ* reporter. In wild-type (H and magnified in J), *Twine-lacZ* expression is found in spermatocytes (arrow in J) and in spermatids. In *pex10* mutants (I and magnified in K), only a few faint signals can be found, in some spermatocytes (arrow in K). (F–I) are at the same magnification. Scale bar: 100 μm.
0.7 and 0.8 ± 0.7 Boule-positive cysts in wild-type and pex10 mutants, respectively (Student’s T-test $P < 0.001; N = 15$). Boule is necessary for translation and accumulation of the Cdc25 phosphatase Twine, which activates Cdc2 to initiate the G2-M transition (25). Similar to Boule staining, there are many more Twine-lacZ translational reporter-positive spermatocytes in wild-type than pex mutants (wt: 17 ± 5 spermatocytes/testis; pex10: 5 ± 2 spermatocytes/testis, $N = 15$ and Student’s T-test $P < 0.001$) (Fig. 3H–K). These results indicate that the majority of pex spermatocytes do not develop to the Boule- and Twine-positive late G2 phase, consistent with the don juan in situ hybridization results.

For each cyst in the testis, there are two cyst cells enclosing the germ cells. We found that the male sterile and spermatocyte developmental phenotypes of pex2 but not pex10 mutants could be fully rescued by germline-specific expression of the UAS-pex2 transgene using either a nos-Gal4 or a hsp83-Gal4 driver. In contrast, expression of UAS-pex2 using the cyst cell-specific Gal4 driver, ptc-Gal4, could not rescue the pex2 mutant phenotype. Together, these results suggest a cell-autonomous role for pex2 during spermatogenesis and distinct roles for pex2 and pex10.

**Other pex mutants have a similar spermatogenesis phenotype**

Since PEX2, PEX10 and PEX12 form a complex during peroxisomal protein import, we expected that loss of function of any of them would lead to a similar phenotype. To address whether the male sterile phenotype is specific to pex2, pex10 and pex12 mutants, we examined other pex gene mutants. PEX1 is an AAA-type ATPase and PEX13 contains an SH3 domain, and both of them are required for peroxisome matrix protein import (26,27). The P-element insertion mutant pex1s4868 is lethal when homozygous, but in trans to a chromosome deficiency it is viable and male sterile. Similarly, the P-element insertion mutant pex13KG04339 is viable but male sterile in trans to a chromosome deficiency. Both alleles are partial loss-of-function alleles with reduced RNA levels, based on RT–PCR results (data not shown). In squashed testis preparations, spermatocytes as well as elongated spermatid bundles were present in young pex1 and pex13 mutants (Fig. 4A and C). However, at the onion stage, most round spermatids were abnormal in both mutants. In wild-type, each bright nucleus is paired with a dark mitochondrial derivative. In pex1 and pex13 mutants, we found that spermatids usually had four nuclei and one large mitochondrial derivative (Fig. 4E, F and G), indicating a cytokinesis defect. This phenotype is reminiscent of the spermatid phenotypes in pex2 and pex10 mutants (Fig. 4G). Moreover, as with pex2 and pex10 mutants, the pex1 and pex13 testis phenotypes progressively worsened with age. In older mutant animals, the cotton ball-like spheres were also present (Fig. 4B and D). PEX16 is required for peroxisome membrane protein targeting (28) and we found that a P-element insertion partial loss-of-function mutant pex16EY05323 also exhibited a cytokinesis defect (Fig. 4G). It is likely due to partial loss-of-function mutations that pex1, pex13 and pex16 mutants have weaker cytokinesis defects than pex2, pex10 and pex12 mutants. These results suggest that the disruption of general peroxisomal function in Drosophila leads to spermatogenesis defects. pex genes appear to be required for at least two stages of spermatogenesis, the spermatocyte G2 phase and spermatocyte cytokinesis.

**PTS1 protein import is defective in pex mutants**

PEX2 and PEX10 are required for the import of PTS1-containing proteins into peroxisomes in mammals and yeast (8,9). To determine whether PEX2 and PEX10 are required for peroxisomal protein import in Drosophila, we
we were able to examine peroxisomal structure in pex10 mutants (Fig. 5H and I). This result is consistent with previous findings that pex10 is required for peroxisomal matrix protein import but not for peroxisomal membrane protein import. Similar studies from other organisms also revealed that the rudimentary structure of peroxisomes is preserved when matrix protein import is defective (30).

**VLCFA accumulates in pex mutants**

In mammals, peroxisomes are essential for β-oxidation in the degradation of VLCFA. In PBD patients, elevated VLCFA is the hallmark of the disease. To address whether Drosophila pex mutants also have the VLCFA accumulation phenotype, we analyzed both LCFA and VLCFA contents in wild-type and pex10 mutant flies using gas chromatography and mass spectrometry (GC/MS). We found that in pex10 mutant males the LCFA (C16 and C18) contents were comparable with wild-type. In pex10 mutants the levels of various VLCFAs, particularly C24 and C26, were higher than in wild-type (Fig. 6A and B). For example, in 2-day-old males, pex10 mutants had 2.5 times more C24 and 2.9 times more C26 than wild-type (Student’s T-test \( P < 0.001 \)). Moreover, in pex10 mutants the VLCFA accumulation phenotype worsened as the animals aged. In 15-day-old animals the accumulation of C24 and C26 increased by 2.6- and 3.9-fold, respectively (Student’s T-test \( P < 0.001 \)) (Fig. 6B). Interestingly, in 15-day-old mutant animals, the levels of C20 and C22 decreased slightly to 0.78- and 0.87-fold, respectively. The progressive increase in VLCFA accumulation may account for the progressively worsening testis phenotype.

VLCFAs residing in the cellular membrane are implicated in influencing membrane properties including the stability of high membrane curvature and membrane fluidity (7,31). Chinese hamster ovary cells lacking pex5 function have reduced membrane fluidity (32). To address how elevated VLCFA levels in pex mutants may contribute to the spermatogenesis phenotype, we measured the membrane fluidity of spermatocytes using fluorescent recovery after photobleaching (FRAP) with a fluorescent probe FM1-43. This technique involves incorporating a fluorescent probe into the cell membrane, photobleaching a small area of the cell under a microscope, and measuring the rate at which fluorescence is recovered as unbleached probe molecules diffuse into the bleached area. We found that after photobleaching wild-type spermatocytes, FM1-43 fluorescence recovery reached a plateau corresponding to \( \sim 83\% \) of the initial fluorescence, with a mean half-time of recovery of \( \sim 21 \) s (Fig. 6C and D). In pex10 mutants, the fluorescence recovery ratio was significantly decreased to \( \sim 67\% \) (Student’s T-test \( P < 0.01 \) compared with wild-type). The mean half-time of recovery was slightly increased to \( \sim 25 \) s (Student’s T-test \( P < 0.05 \) compared with wild-type) (Fig. 6D and E). Together these findings indicate that Drosophila pex mutants are defective in the degradation of VLCFA, which may subsequently influence the membrane properties of spermatocytes, causing defective spermatocyte development.

generated PTS1-containing GFP transgenes by adding the three amino acid prototype PTS1 signal SKL to the tail of GFP and examined GFP-SKL expression patterns in wild-type and pex mutant animals (29). In all tissues examined including imaginal disc, midgut, salivary gland and testis, numerous fine dots, presumably peroxisomes, were observed in wild-type (Fig. 5A and C). However, when the GFP-SKL transgene was introduced into either pex2 or pex10 mutants, the GFP signals were no longer punctate. Instead the signal was diffuse and sometimes located in the nucleus (Fig. 5B and D). Therefore peroxisomal matrix protein import is likely to be impaired in Drosophila pex mutants.

To further reveal peroxisomal structures, we created a PEX2::mCherry transgene. The fusion protein is functional because it fully rescued the male sterile phenotype of pex2 mutants when expressed in testes. In wild-type, PEX2::mCherry colocalized with the peroxisomal marker GFP::SKL (Fig. 5E–G, indicating that it is localized to peroxisomes. Since the expression of PEX2::mCherry could not rescue the male sterile phenotype in pex10 mutants (data not shown), we were able to examine peroxisomal structure in pex10 mutants using the PEX2::mCherry marker. In pex10 mutants, the distribution and brightness of PEX2::mCherry appear similar to wild-type. Therefore, although peroxisomal matrix protein import was impaired, peroxisomal membrane protein targeting and peroxisomal structures appeared quite normal in pex10 mutants (Fig. 5H and I). This result is consistent with previous findings that pex10 is required for peroxisomal matrix protein import but not for peroxisomal membrane protein import. Similar studies from other organisms also revealed that the rudimentary structure of peroxisomes is preserved when matrix protein import is defective (30).
The pex mutant phenotype can be enhanced by increasing the VLCFA level

To further address whether accumulation of VLCFA is a causative factor for the spermatocyte development defects in pex mutants, we altered the VLCFA level in the fly food and then examined the consequence for spermatogenesis. Wild-type testes appeared the same after these treatments. Interestingly, when we grew pex10 mutants on medium to which we had added 2.5% VLCFA (C22:0, C22 is the length of carbon chain and 0 is the number of double bond or C24:0), the testis phenotype was significantly enhanced compared to pex10 flies grown on non-supplemented food (Fig. 7A–F; data not shown). As shown in Figure 7, the testes of the VLCFA-supplemented mutants appeared much smaller and contained fewer cells at eclosion (Fig. 7B). Moreover, the phenotype became more severe in just a week (Fig. 7E). The enhancement of the testis phenotype by VLCFA is even more robust in pex13 partial loss-of-function mutants. In pex13 mutants, the elongated spermatid bundles that are usually present under normal food conditions disappeared when the flies were fed VLCFA-supplemented food. Instead, cotton ball-like structures were present, resembling the phenotype of homozygous pex2 null flies fed on normal food (Fig. 7C and F). In contrast, adding 2.5% LCFA (C18:1 or C16:0) to the food did not enhance the testis phenotype (Fig. 7G–L; data not shown), indicating that the pex mutant phenotype was specifically affected by VLCFA but not LCFA. Since VLCFA levels are increased in pex mutants, it is likely that accumulation of VLCFA causes the spermatocyte development defects of pex mutants.

pex Mutants genetically interact with VLCFA biosynthetic mutants

Since the degradation of VLCFA is impaired in pex mutants, and exogenous VLCFA could further enhance pex phenotypes, we tested whether pex mutants could influence VLCFA biosynthesis-related mutants, or vice versa. VLCFA biosynthesis is achieved through repeated rounds of the two-carbon addition process from LCFA by multiple enzymes, including a synthase/elongase, a dehydratase and two reductases (Fig. 8D) (31). We first tested the genetic interaction between pex and bond, which encodes one of 20 predicted VLCFA elongases in Drosophila. bond mutants were reported to have a male sterile phenotype with cytokinesis defects (Fig. 8A) (33). Interestingly, we found that eliminating one copy of either the pex2 or pex10 gene partially suppressed the male sterile and cytokinesis defects of bond mutants. The suppression was more prominent when one copy of pex2 and one copy of pex10 were removed.
simultaneously (Fig. 8A and B), suggesting that having the proper amount of VLCFA is critical for cytokinesis.

Sc2 encodes the only enoyl reductase in *Drosophila*, which catalyzes the last step in each cycle of VLCFA elongation (31). When Sc2 function is compromised, VLCFA biosynthesis is likely to be blocked. Since the bond mutation could not alleviate the mutant phenotype of *pex2* or *pex10* null mutants (data not shown), we reasoned that partial loss of function of *pex* might be helpful in revealing sensitive genetic interactions. A hypomorphic allele of *pex13* (*pex13KG04339*) was used to examine the effects of over-expression of Sc2 or partial loss of Sc2 function on *pex* mutants. Sc2 is a putative null mutant, and is larval lethal. Sc2 is a UAS element insertion allele and on its own it represents a weak loss of function allele of Sc2, but when in combination with a Gal4 driver, it can be used to over-express Sc2. We found that over-expression of Sc2 with the germine-specific nos-Gal4 driver enhanced the *pex13* cytokinesis defect (Fig. 8C). In contrast, when we combined the Sc2 trans-heterozygote with the *pex13* mutation, the cytokinesis defect of *pex13* was partially suppressed (Fig. 8C). Together, these results imply that the VLCFA level is critical for *Drosophila* spermatogenesis and the accumulation of VLCFA results in spermatocyte development defects in *pex* mutants.

**DISCUSSION**

In this study, we showed that *Drosophila* pex mutants faithfully recapitulate several key features of human PBD: impaired peroxisomal protein import, elevated VLCFA levels and growth retardation. The most obvious phenotype of *Drosophila* pex mutants is male infertility. In agreement with our studies, conditional knockouts of *pex5* and *pex7* in mice also led to male sterility, and a male patient with the VLCFA accumulation disease ALD was found to have a rapid decline in fertility (34–36). Thus, progressive loss of male fertility may be another conserved feature of PBD.

**Peroxisomes and spermatogenesis**

We have traced the male sterile phenotype of *Drosophila* pex2 and pex10 mutants to abnormal spermatocyte development. Since peroxisomes are presumably present in every type of cell, why do *Drosophila* pex mutants exhibit profound phenotypes specifically in testis? One possible reason is that the dramatic changes in cellular and subcellular morphology during spermatogenesis require a large supply of membrane, rendering spermatogenesis extremely vulnerable to subtle membrane alterations. To accomplish the cell size increase during the spermatocyte growth phase and the membrane remodeling process during spermatid differentiation, membrane synthesis and dynamics have to be precisely regulated during spermatogenesis. Deficiencies in genes that function in intracellular membrane trafficking, such as *Arf6*, *Syx5* and *Cog-5*, often result in spermatid differentiation defects (37–39). Therefore, it follows that defect in VLCFA lipid metabolism in pex mutants may lead to subtle membrane abnormalities, which may result in defects in spermatocyte development. In contrast, in most somatic cells, the subtle abnormalities may only lead to mild or non-detectable phenotypes.

*Drosophila* pex mutants exhibit a strong meiotic cytokinesis defect. Similarly, the testes of *pex7*;*Abcd1* double mutant mice
were reported to have multinucleated cells and to lack normal haploid round spermatids, a phenotype that is likely due to cytokinesis defects (40). Since pex mutants are viable, it is unlikely that pex mutants display an equally strong mitotic cell division defect. Why do pex mutations specifically affect meiotic cell division? There are many other genes that, when mutated, affect meiotic cytokinesis but not mitotic cytokinesis. There are at least two possible explanations for such phenomena. First, somatic tissues may have compensatory gene functions not present in germ cells, accounting for the observed testis-specific and meiotic cytokinesis-specific defects in mutants. For example, one phosphatidylinositol 4-kinase, Fwd, regulates actin organization during meiotic cytokinesis (41). Secondly, the spermatocyte meiotic cell division may be more vulnerable to loss of pex function than mitotic cell division due to the characteristic features of meiosis such as two consecutive rounds of cell division or rapid furrow ingression. Therefore, defects in a general cellular process or partial loss-of-function mutations of essential genes might lead to a meiotic cytokinesis defect but little or no mitotic cytokinesis defect. For example, mutations of essential genes might lead to a meiotic cytokinesis defects in a general cellular process or partial loss-of-function rounds of cell division or rapid furrow ingression. Therefore, defects in a general cellular process or partial loss-of-function mutations of essential genes might lead to a meiotic cytokinesis defect but little or no mitotic cytokinesis defect. For example, partial loss-of-function mutation of the Drosophila rab11 gene causes abnormal accumulation of Golgi-derived vesicles and defective actomyosin ring constriction during meiotic cytokinesis (42). Since peroxisomes are present in most, if not all, cells and pex mutants should affect peroxisomal functions in general, we believe that the cytokinesis defects found in pex mutants may reflect the latter possibility.

**VLCFA levels and the severity of mutant phenotype**

Peroxisomes participate in the metabolism of fatty acids, particularly VLCFA in animals. Fatty acids are key constituents of cellular phospholipids. The composition of fatty acids in the phospholipids along with membrane cholesterol content determines the physiological properties of membranes. LCFA (C16 and C18) are the most abundant fatty acids in membranes. VLCFA levels are usually low in most tissues. However, in certain specialized animal tissues, such as the retina, spermatooza, and the myelin sheath, VLCFAs are present in high levels (7). Therefore, it is possible that differences in VLCFA levels may reflect the tissue-specific roles of VLCFA in different biological processes.

Indeed, VLCFA has been recently implicated in Drosophila sperm development. bond encodes a VLCFA elongase protein which is important for VLCFA biosynthesis (Fig. 8D). bond mutants presumably have low VLCFA levels or a scarce supply of a particular VLCFA and/or its derivatives. bond mutants display strong cytokinesis defects characterized by slow cleavage furrow ingression, usually followed by regression (33). Interestingly, we found that mutations in pex genes, which have elevated VLCFA, also lead to cytokinesis defects, indicating that the proper level of VLCFA is important for spermatocyte cytokinesis. Dosage-dependent suppression of bond by pex mutants and the genetic interaction between pex13 with Sc2 are consistent with this conclusion. Furthermore, our studies reveal three possible outcomes of disrupted VLCFA homeostasis. High levels of VLCFA result in severe spermatocyte development defects. Moderately high levels of VLCFA cause cytokinesis defects during meiosis. Lower than normal levels of VLCFA also lead to defective meiotic cytokinesis. The genetic interaction of pex13 with Sc2 and the enhancement of the pex mutant phenotype by exogenous VLCFA point to a causative link between VLCFA levels and the severity of the mutant phenotype. Thus, maintaining a proper level of VLCFA is crucial for normal physiological conditions in developing sperm.

So far, the specific roles of VLCFAs during spermatogenesis remain a mystery. Besides playing a structural role in cellular membranes, VLCFAs could have metabolic and signaling roles, such as being components or precursors of ceramide, sphingolipid and glycosylphosphatidylinositol anchors (7). Why both elevated and reduced levels of VLCFAs lead to the same cytokinesis defects is currently unknown. Decreased membrane fluidity caused by increased VLCFA levels might interfere with the rapid membrane ingression during cytokinesis. Disturbing membrane ingression may lead to cytokinesis defects. Further analysis will be required to pin down the particular roles of VLCFA in spermatogenesis and to gain more insight into the disease mechanism of PBD. Nevertheless, our results indicate that pex genes are essential for maintaining proper levels of VLCFA. Moreover, VLCFAs play important roles in spermatogenesis.

In addition to loss of male fertility, another prominent feature of PBD is mental retardation associated with defective myelin sheaths. Oligodendrocyte specific knockout of pex5 in mice also results in axonal loss and demyelination (16,43). Although the exact mechanism underlying the neuronal phenotype is not known, it is likely that altered VLCFA content in the membrane might be involved, because VLCFAs and their derivatives are the predominant lipid constituents of the membranous myelin sheath. Although we have not analyzed neuronal morphology in detail, Drosophila pex mutant flies have a normal life span with no overt behavioral defects (data not shown). It is unclear why Drosophila pex mutants behave normally whereas mice and humans are severely affected, but there may be critical differences in neurobiology between different species. For example, Drosophila neurons lack a myelin sheath. Therefore, the nervous system in Drosophila pex mutants may be more mildly affected and the neuron degeneration phenotype could have a late onset. For example, in flies mutant for the bubblegum gene (encoding a VLCFA acyl-CoA synthetase), the VLCFA level is elevated as seen in human ALD and PBD patients and the mutants display late onset neuron degeneration (44).

The results from our studies show that the pex mutants in Drosophila can serve as a genetic model for PBD. In particular the observation that VLCFA levels influence the severity of pex mutant phenotypes makes us believe that pex mutants could be promising tools for drug development as well as genetic screening for potential modulators. Chemical compounds or genetic modulators that can suppress or partially suppress the mutant phenotypes could be potential candidates for developing intervention strategies for PBD.

**MATERIALS AND METHODS**

**Drosophila culture and stocks**

Flies were cultured on standard cornmeal food at 25°C unless otherwise specified. The following alleles and transgenes were used in this study: pex2^{HP3509}, pex2^{01899}, pex10^{10}, pex10^{10}, pex12^{01300}, pex1^{5868}, pex13^{EG439}, bond^{5224}, bond^{Z3457},
Molecular biology

UAS-pex2 was created by amplifying full-length pex2 cDNA from cDNA clone LD46714 and subcloning it into the EcoRI–KpnI sites of pUAST. The stop codon of pex2 was replaced with a KpnI site using PCR and then full-length pex2 cDNA without a stop codon was inserted in frame into a mCherry vector. Similarly, (mCherry cDNA without a stop codon was inserted in frame into a pAc5.1-GFP::SKL vector. The pex2::mCherry fusion fragment (EcoRI–XbaI) was cloned into a pUAST-attB vector to create the UAS-pex2::mcherry construct. pCasper4-act-GFP::SKL was generated by inserting a BglII–HpaI fragment of pAc5.1-GFP::SKL (29) into BamHI–HpaI-cut pCasper4 vector. Similarly, pCasper3-β2tub-GFP::SKL was generated using KpnI–NotI digestion to place a GFP-SKL fragment into a pCasper3-β2tub vector (45). The sequences of all constructs requiring PCR amplification were confirmed by sequencing. RT–PCR to assess gene expression in various mutants was performed with primers corresponding to each gene. Primer sequences are available upon request.

Immunostaining and microscopy

Testes from 1 to 2-day-old animals were used in immunostaining and phenotypic quantification. Testis squashes for phase contrast microscopy and immunolabeling were performed as described previously (23). In situ hybridization and β-galactosidase activity stains were performed as previously described with minor modifications (20). A 1:200 dilution of BrdU (BD, Franklin Lakes, NJ, USA) was used in pulse labeling. Primary antibody incubations were overnight at 4°C with anti-Cyclin A (Kindly provided by Z. Wang) 1:2000 (22,46) and anti-Boule (Kindly provided by S. Wasserman) 1:100 (25). Images were captured using a Zeiss microscope. P-values were calculated with two-tailed Student’s T-test.

Free fatty acid analysis

Total fat extracts of 100 male flies were used for the methylation of fatty acids (44). Gas chromatography was performed using a HP-5 column (30 m by 0.25 mm, 0.25 μm thickness), and mass spectrometry was used to identify and quantify fatty acid concentrations, with C27 added as an internal standard. Each group was repeated at least three times.

Quantification of cytokinesis defects and male fertility test

Squashed testes from 2-day-old males were used to quantify cytokinesis defects using phase contrast microscopy. For each genotype, testes from 15 males were scored. For male fertility tests, one young male was crossed to three virgin females for 5 days. The adults were then removed and 7 days later the number of eclosing adults and unhatched pupae were recorded.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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