

A *Drosophila* model for the Zellweger spectrum of peroxisome biogenesis disorders

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SUMMARY

Human peroxisome biogenesis disorders are lethal genetic diseases in which abnormal peroxisome assembly compromises overall peroxisome and cellular function. Peroxisomes are ubiquitous membrane-bound organelles involved in several important biochemical processes, notably lipid metabolism and the use of reactive oxygen species for detoxification. Using cultured cells, we systematically characterized the peroxisome assembly phenotypes associated with dsRNA-mediated knockdown of 14 predicted *Drosophila* homologs of PEX genes (encoding peroxins; required for peroxisome assembly and linked to peroxisome biogenesis disorders), and confirmed that at least 13 of them are required for normal peroxisome assembly. We also demonstrate the relevance of *Drosophila* as a genetic model for the early developmental defects associated with the human peroxisome biogenesis disorders. Mutation of the *PEX1* gene is the most common cause of peroxisome biogenesis disorders and is one of the causes of the most severe form of the disease, Zellweger syndrome. Inherited mutations in *Drosophila Pex1* correlate with reproducible defects during early development. Notably, *Pex1* mutant larvae exhibit abnormalities that are analogous to those exhibited by Zellweger syndrome patients, including developmental delay, poor feeding, severe structural abnormalities in the peripheral and central nervous systems, and early death. Finally, microarray analysis defined several clusters of genes whose expression varied significantly between wild-type and mutant larvae, implicating peroxisomal function in neuronal development, innate immunity, lipid and protein metabolism, gamete formation, and meiosis.

INTRODUCTION

Peroxisome biogenesis disorders (PBDs) are a broad spectrum of human diseases originating from mutations in genes encoding proteins responsible for the formation and maintenance of peroxisomes. Peroxisome number, shape, size and protein composition vary dramatically depending on the cell type and environment. In mammals, peroxisomes are particularly abundant in the liver, kidneys and central nervous system (CNS), where they are found primarily in the oligodendroglia. Peroxisomes contain more than 50 different enzymes that are involved in a variety of important metabolic pathways, including the oxidative breakdown of fatty acids, peroxide detoxification, the oxidation of L- and D-amino acids and polyamines, and the synthesis of plasmalogens, bile acids, ether phospholipids and polyunsaturated fatty acids [for classical and current views of peroxisomes and their functions, see the following references (Lazarow and Fujiki, 1985; van den Bosch et al., 1992; Wanders and Waterham, 2006; Schrader and Fahimi, 2008; Mast et al., 2010)].

The contribution of peroxisome function to normal human development and physiology is underscored by the severity and lethality of PBDs. PBDs are a heterogeneous group of fatal

autosomal recessive diseases that includes Zellweger syndrome (ZS), rhizomelic chondrodysplasia punctata (RCDP), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). These four disorders are classified on the basis of the severity of clinical phenotypes. In addition to these broad biogenic disorders, there are additional single-enzyme peroxisome diseases that share many of the same traits as these more serious disorders. In general, patients with PBD suffer from profound neurological abnormalities, muscular hypotonia, cataracts, cardiac defects, dysmorphic features, and growth and mental retardation (for reviews, see Shimozawa et al., 2005; Steinberg et al., 2006). ZS is the most severe and typically presents with severe hypotonia, such that children with ZS are sometimes initially misdiagnosed with various forms of developmental, mental and physical retardation, including Down syndrome, Prader-Willi syndrome or spinal muscular dystrophy. Patients with ZS typically die in the first year of life (Wilson et al., 1988; Yik et al., 2009). The overall incidences of the PBDs vary widely among different population groups but are invariably infrequent enough to be classified as rare genetic diseases. ZS has an estimated incidence of 1 in 50,000 in North America but approximately 1 in 500,000 in Japan (Steinberg et al., 2004).

The molecular mechanisms underlying peroxisome biogenesis have been strongly conserved during evolution (Platta and Erdmann, 2007; Schrader and Fahimi, 2008). To date, 34 PEX genes encoding peroxins, or proteins required for peroxisome assembly, have been identified (Platta and Erdmann, 2007; Mast et al., 2010; Tower et al., 2011). Of these, 13 PEX genes functionally complement the defects in peroxisome assembly of all the known complementation groups of the PBDs (Shimozawa et al., 2005; Steinberg et al., 2006). These 13 PEX genes function in a variety of different steps in the peroxisome biogenic pathway, including peroxisomal membrane assembly and peroxisomal protein

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targeting. An unexpected complication in the assignment of PBD phenotypes to any particular complementation group is the fact that different complementation groups share similar clinical phenotypes (Moser et al., 1995; Steinberg et al., 2006). This is most probably due to the fact that a defect in any aspect of the peroxisome biogenic program results in the same functional loss of the organelle. As a result, clinical diagnosis of the cause of any one PBD has required an in-depth and tedious biochemical characterization followed by sequencing efforts to pinpoint the source of the mutation (Steinberg et al., 2004; Shimozawa, 2011). Advances in sequencing technology have improved the speed of diagnosis and there is currently an undertaking to fully annotate and catalog the range of mutations present along the PBD continuum (<http://dbpex.org/home.php>). In addition, model systems have greatly aided the identification and characterization of peroxisomal genes (Titorenko and Rachubinski, 2001).

Although single-cell systems such as yeast and mammalian cell culture have been invaluable for identifying the proteins and basic processes involved in peroxisome biogenesis, they cannot act de facto as models for the study of the role of peroxisomes in the development of multicellular organisms or in the molecular mechanisms leading to PBDs. *Drosophila* has been shown to be both a valid and valuable model system for the study of normal and abnormal development of more complex multicellular organisms, including humans. Over 75% of known human disease genes have a recognizable match in the genome of *Drosophila* (Reiter et al., 2001), and *Drosophila* now serves as a model for more than 20 diseases, including Huntington's, Parkinson's, Alzheimer's, fragile X mental retardation and spinocerebellar ataxia 1 (Bilen and Bonini, 2005; Cauchi and van den Heuvel, 2006; Raymond and Tarpey, 2006; Sánchez-Martínez et al., 2006). Surprisingly, there have been few studies of the functional roles of peroxisomes in *Drosophila* and, until this study, none focused

on the effects of peroxisome function on early development. The longest-known role of peroxisomes in flies is in the formation of eye pigments. The *rosy* (*ry*) gene encodes the enzyme xanthine dehydrogenase (Bridges and Brehme, 1944; Glassman and Mitchell, 1958), which is targeted to peroxisomes in Malpighian tubule cells and fat cells (Beard and Holtzman, 1987). Recent studies have also suggested a role for peroxins in the process of spermatogenesis in adult males (Chen et al., 2010). However, no study has focused specifically on the functional roles of peroxisome biogenesis (Pex) genes of *Drosophila melanogaster* or on the defects in early fly development arising from mutation of a Pex gene, and the similarity between those defects and the developmental defects observed in PBD patients mutant for the corresponding human PEX gene.

To first establish a *Drosophila* model for the embryonic development defects associated with the PBDs, we performed a systematic double stranded (ds)RNA knockdown screen in embryonically derived Schneider 2 (S2) cells to confirm homologous roles of the 14 *Drosophila* genes homologous to known PEX genes in other organisms (Fig. 1) (Chen et al., 2010). Importantly, 12 of the PEX genes have known mutations that lead to PBD formation. Once we had demonstrated that the majority of *Drosophila* Pex proteins are required for peroxisome assembly, we next assayed the functional requirement for peroxisomes in *Drosophila* embryonic development by examining the developmental phenotype of flies with homozygous mutations in *Pex1*, because mutations in human *PEX1* are the most common cause of PBD (Reuber et al., 1997; Steinberg et al., 2006). *Pex1* mutant larvae exhibit abnormalities that are analogous to those observed in ZS patients. These abnormalities correlate to reproducible changes in gene expression between larvae lacking functional *Pex1* and wild-type larvae. Our results support the use of *Drosophila* for studying the role of PEX genes in early development and as a genetic model for PBDs and, in particular, ZS.

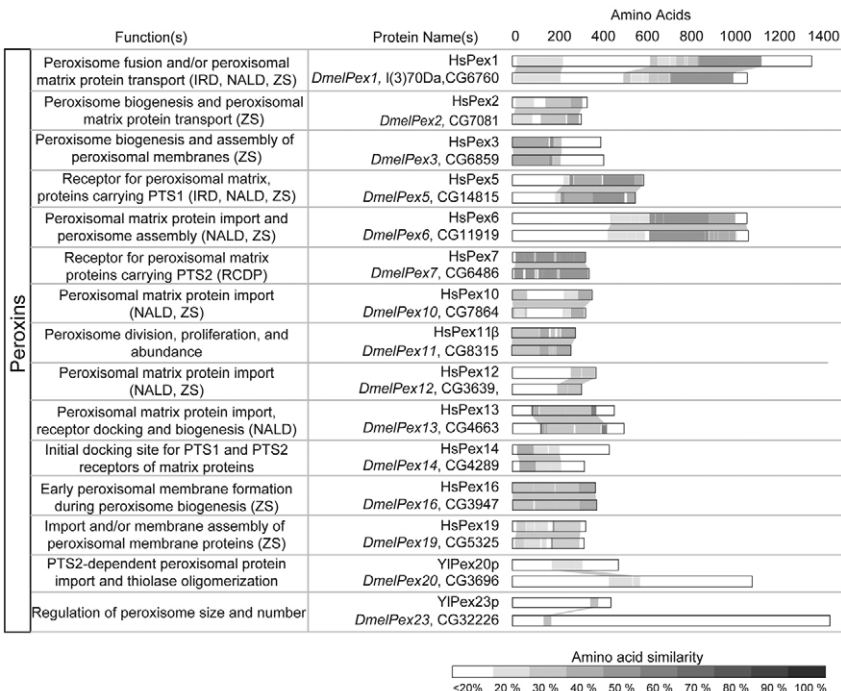


Fig. 1. Peroxins and their putative homologs in *Drosophila*. The putative *Drosophila* homologs of known peroxins that we identified in silico are presented. The main function in peroxisome biogenesis of each known peroxin is given. The PBD in which a PEX gene has been implicated is indicated in brackets. Pairwise alignment is made between the human or yeast peroxin (upper) and the putative *Drosophila* homolog (lower) using the SIM alignment algorithm (<http://ca.expasy.org/tools/sim-prot.html>) and visualized using Lalnview (<http://pbil.univ-lyon1.fr/software/lalnview.html>). The extent of amino acid similarity between regions of two aligned proteins is given by the Heat map at bottom. Hs, *Homo sapiens*; Yl, *Yarrowia lipolytica*; Dmel, *Drosophila melanogaster*.

RESULTS

RNAi analysis in S2 cells confirms that the majority of the known genes required for peroxisome assembly are conserved in *Drosophila*

Fifteen putative Pex genes are predicted in the *Drosophila* genome. Thirteen genes are homologous to known human PEX genes, whereas two genes are homologous to the *PEX20* and *PEX23* genes of the yeast *Yarrowia lipolytica* (Fig. 1). These *Drosophila* Pex genes correlate with genes predicted by others as *Drosophila* homologs for PEX genes found in other species (Adams et al., 2000; Chen et al., 2010). Twelve of these genes are homologous to known human PEX genes for which mutations causing PBDs have been characterized.

Mutations in PEX genes typically result in the mislocalization of peroxisomal matrix proteins to the cytosol, and this mislocalization of matrix proteins is used along with other biochemical laboratory diagnostics in characterizing a PBD (Shimozawa et al., 1999; Steinberg et al., 2006; Shimozawa, 2011). We performed systematic dsRNA interference (RNAi) knockdown

of each Pex gene in S2 cells constitutively expressing a chimeric reporter protein, GFP-SKL. The SKL tripeptide is the evolutionarily conserved C-terminal peroxisome targeting signal 1 (PTS1), consisting of Ser-Lys-Leu (SKL) that targets specifically to peroxisomes to produce a characteristic punctate pattern in fluorescence microscopy (Petriv et al., 2002; Kural et al., 2005). GFP-SKL-expressing S2 cells were treated with dsRNAs targeting 14 individual putative *Drosophila* Pex genes for knockdown: *Pex1* (CG6760), *Pex2* (CG7081), *Pex3* (CG6859), *Pex5* (CG14815), *Pex6* (CG11919), *Pex7* (CG6486), *Pex11* (CG8315), *Pex12* (CG3639), *Pex13* (CG4663), *Pex14* (CG4289), *Pex16* (CG3947), *Pex19* (CG5325), *Pex20* (CG3696) and *Pex23* (CG32226) (Fig. 1). As a control for specificity of the RNAi knockdown, we performed a mock RNAi treatment (no dsRNA), as well as an RNAi knockdown of *Dredd*, which has no known role in peroxisome biogenesis or function.

Untreated GFP-SKL S2 cells exhibited a punctate pattern of fluorescence, which is characteristic of peroxisomes (Fig. 2) (Kural et al., 2005). Similarly, in mock-treated and *Dredd* RNAi-treated

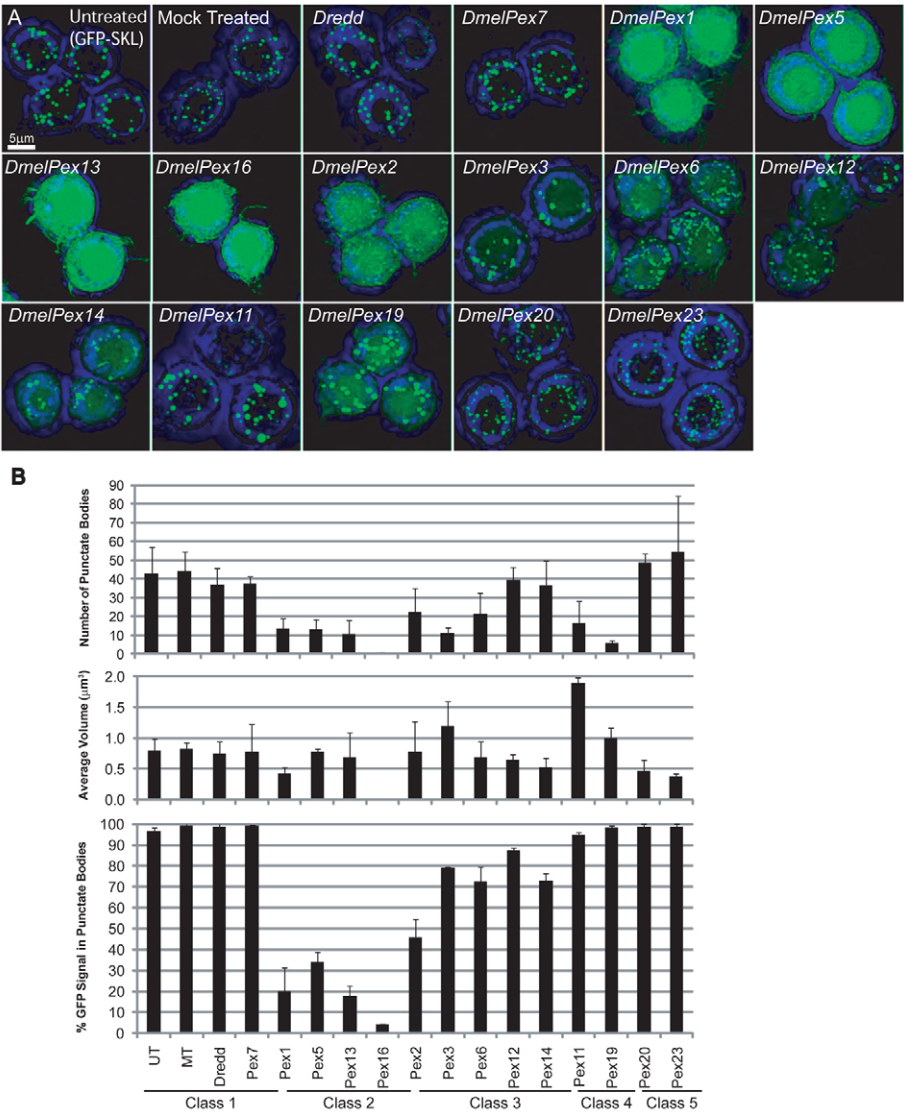


Fig. 2. Peroxisomes are absent or exhibit altered morphology in S2 cells treated with dsRNA to putative Pex genes. (A) S2 cells constitutively expressing the fluorescent peroxisomal reporter protein GFP-SKL (Kural et al., 2005) were treated with dsRNA to the indicated putative Pex genes, mock-treated (MT) or treated with dsRNA targeting *Dredd*, which has no known role in peroxisome biogenesis or peroxisome function. GFP-SKL in S2 cells targets to punctae that are characteristic of peroxisomes. Mock-treated cells and cells treated with dsRNA targeting *Dredd* exhibited punctae in the same pattern as control cells (UT). Cells treated with dsRNAs to different Pex genes exhibited mislocalization of the GFP-SKL peroxisomal reporter to the cytosol and/or altered peroxisomal size and number. Cells treated with dsRNA to *Pex7* or *Pex20* exhibited punctate peroxisomes that were essentially like those of wild-type cells, because *PEX7* and *PEX20* affect the targeting only of peroxisomal proteins containing a PTS2 and not of those containing PTS1, such as GFP-SKL. (B) Quantitative description of peroxisome morphologies in S2 cells treated with dsRNA to putative Pex genes. Images were scored for numbers of punctate bodies, the average volume of these punctate bodies and the intensity of fluorescent signal from these punctate bodies.

cells, the GFP-SKL signal also appeared in punctate structures, consistent with peroxisome localization. The peroxisomes in control and untreated cells were spherical, of essentially uniform size and randomly distributed throughout the cell.

In S2 cells, RNAi knockdown of many of the putative *Drosophila* Pex genes that we identified (supplementary material Fig. S1 and Table S1) resulted in GFP-SKL fluorescence patterns that were significantly different from the punctate fluorescence pattern observed in untreated or control-treated cells (Fig. 2A). We grouped these patterns into five classes (Fig. 2B). Class 1 cells exhibit a pattern of GFP-SKL fluorescence that is essentially indistinguishable from that of untreated cells. Mock-treated and *Dredd* RNAi-treated cells were categorized as Class 1, as were cells treated with dsRNA targeting *Pex7*. This was not unexpected, because Pex7p has been shown to function in the targeting to peroxisomes of proteins containing an N-terminally localized PTS2 but not of proteins containing a C-terminally localized PTS1 (Lazarow, 2006) (i.e. the chimeric reporter GFP-SKL being expressed in these S2 cells). In Class 2 cells, most of the GFP signal was excluded from punctate bodies. Putative Pex genes in Class 2 include *Pex1*, *Pex5*, *Pex13* and *Pex16*. Class 3 cells show an appreciable amount of cytosolic GFP signal but contain fluorescent punctate bodies in numbers approaching those of untreated cells and were observed in RNAi knockdown of *Pex2*, *Pex3*, *Pex6*, *Pex12* and *Pex14*. Class 4 cells have a reduced number of fluorescent punctate bodies of increased size as compared with untreated cells but show no appreciable accumulation of GFP signal in the cytosol. RNAi knockdown of the *Pex11* and *Pex19* genes produced a Class 4 pattern. In Class 5 cells, the average volume of punctate bodies was less and the average number of punctate bodies per cell was greater than that of untreated cells, and all the GFP signal was essentially localized to punctate bodies. Cells treated with dsRNA to *Pex20* or *Pex23* were categorized as Class 5.

The specificity of transcript knockdown by RNAi was verified for the case of *Pex1* by semi-quantitative reverse-transcriptase PCR (RT-PCR; Fig. 3A) and by immunoblotting of whole S2 cell lysates with antibody to Pex1 protein (Fig. 3B). The levels of *Pex1* mRNA were reduced in GFP-SKL S2 cells by treatment with *Pex1* dsRNA but not by treatment with *Dredd* dsRNA or by mock treatment (Fig. 3A). Pex1 protein was reduced in lysates of S2 cells treated with *Pex1* dsRNA but not in lysates of mock-treated S2 cells or S2 cells treated with *Pex7* dsRNA (Fig. 3B). The results of our RNAi analysis in S2 cells demonstrate that loss of function in the majority (13 of 14) of putative Pex genes identified in silico produces abnormal peroxisome assembly phenotypes in *Drosophila* S2 cells.

Mutation of *Pex1* leads to tissue-specific defects in developing embryos

PEX1 genes in organisms from yeast to human encode members of the family of AAA-ATPases and are essential for peroxisome assembly. Mutation of human *PEX1* results in ZS and is the most common cause of PBD, accounting for 70% or more of all patients (Reuber et al., 1997; Steinberg et al., 2006). Because of the prevalence of the *PEX1* mutation in the PBDs, studies of *Pex1* mutations were a natural starting point for evaluation of a *Drosophila* model for the systemic effects of the PBDs and, more specifically, of ZS.

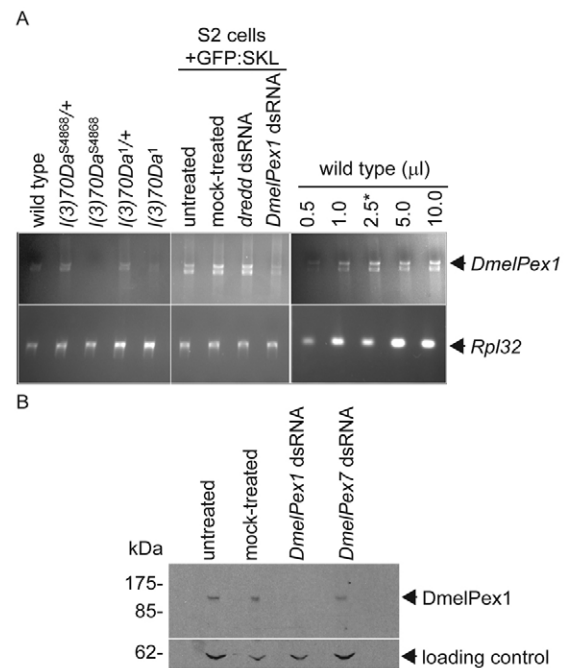


Fig. 3. Detection of *Pex1* transcripts by RT-PCR and of *Pex1* protein by immunoblotting. (A) The specific bands corresponding to the expression levels of *Pex1* and the gene *Rpl32*, encoding a ubiquitously expressed ribosomal protein and used as a loading control, are indicated by arrows. Heterozygous *l(3)70Da* animals are indicated by '+'. The mRNA isolated from *l(3)70Da^{S4648}* homozygotes produced no *Pex1*-specific band, whereas *l(3)70Da¹* homozygous animals showed a severe reduction in this band compared with the *Rpl32* loading control. Similarly, the level of *Pex1* transcript was reduced in the dsRNA-treated S2 cells compared with untreated, mock-treated or S2 cells treated with a dsRNA that targets the *Dredd* gene, which is involved in the immune response. For each set of primers, specific amounts of the wild-type RT-reaction were analyzed to confirm that 2.5 μl of experimental sample yielded a product within the linear range of amplification by the subsequent PCR. (B) *Pex1* protein is reduced specifically in cells treated with dsRNA to the *Pex1* transcript. Lysates of untreated S2 cells, mock-treated S2 cells, S2 cells treated with dsRNA to *Pex1* mRNA and S2 cells treated with dsRNA to *Pex7* mRNA were separated by SDS-PAGE and subjected to immunoblotting with anti-Pex1 protein antibodies. Reduced levels of Pex1 protein are observed only in the lane containing lysate of S2 cells treated with dsRNA to *Pex1* mRNA. A protein detected nonspecifically by the antibodies to Pex1 protein serves as a control for protein loading. Numbers at left represent the migrations of molecular mass standards in kDa.

To study the functional consequences of compromised peroxisome biogenesis in the early development of *Drosophila*, we performed an in-depth phenotypic characterization of how loss-of-function mutations in the *Pex1* gene affect basic tissue patterning, survival and the functional response of the organism in terms of altered gene expression. Both P-element and X-ray-induced mutations in *Pex1*, *l(3)70Da^{S4868}* and *l(3)70Da¹*, respectively, were characterized in terms of defects during early development. These alleles are lethal when homozygous, in trans or when heterozygous to a large deletion, *Df(3L)jz-GS1a*, that removes the chromosomal region 70D2-70E5, which includes the *Pex1* locus. However, we noted that the genomic region encoding

the 3'-UTR of the *Pex1*, *l(3)70Da¹* mRNA overlaps the genomic region encoding the 5'-UTR of the adjacent gene, *breathless (btl)* (Glazer and Shilo, 1991; Adams et al., 2000). The primary phenotype associated with mutation of *btl* is defective tracheal development. No tracheal defects were observed in homozygous *l(3)70Da^{s4868}* (*Pex1^{s4868}*) or *l(3)70Da¹* mutants (data not shown). However, to avoid the possibility of confounding phenotypes, we used only the alleles *l(3)70Da^{s4868}* and *l(3)70Da¹*, which harbor smaller mutations affecting only the 5' region of the *Pex1* gene, for subsequent developmental characterizations. Finally, ubiquitous expression of a UASp-*Pex1* transgene via Tub-GAL4 in the early embryo allowed homozygous *l(3)70Da^{s4868}* and *l(3)70Da¹* mutants to survive past the second larval instar, confirming that the early larval lethality observed in these mutants was caused by loss of *Pex1*.

RT-PCR and immunoblotting confirmed specific reduction in the *Pex1* transcript and protein expression in homozygous mutant *l(3)70Da^{s4868}* and *l(3)70Da¹* larvae 2 days after hatching (Fig. 3A). Heterozygous *Pex1^{s4868}* flies grew (Fig. 4) and survived (Fig. 5) like wild-type flies and, within 2 hours of hatching, both wild-type and heterozygous *Pex1* larvae exhibited the same coordinated locomotory activity in their movement towards a food source (Fig. 6). This is in contrast to animals homozygous for the *l(3)70Da^{s4868}* allele, which grew much more slowly than wild-type or heterozygous flies (Fig. 4) and died at the L1 or L2 stage (Fig. 5). These homozygous mutant larvae were consistently much smaller than wild-type or heterozygous larvae, exhibited little or no coordinated locomotion when placed on agar plates, failed to show effective feeding and exhibited developmental delay (Fig. 6). There is some phenotypic pleiotropy associated with the *l(3)70Da^{s4868}* and *l(3)70Da¹* alleles, and, in extreme cases, homozygous mutants were unable to fully crawl out of their eggshells and died within a few hours of hatching. Overall, our results demonstrate that *Pex1*, like *PEX1* for humans, is essential for normal embryonic development.

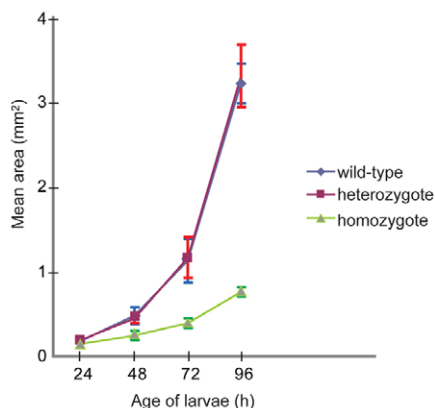


Fig. 4. *Pex1^{s4868}* homozygous larvae exhibit defects in growth. Each data point represents the average size in mm² of 20 randomly selected *Pex1^{s4868}* homozygous (green), *Pex1^{s4868}* heterozygous (purple) and wild-type (blue) larvae. On day 4, the mean area, which represents growth, of *Pex1^{s4868}* homozygous larvae is significantly reduced, as compared with wild-type and heterozygous larvae ($P < 0.0001$). Wild-type and heterozygous larvae did not show any statistically significant difference in growth. Error bars indicate standard deviation.

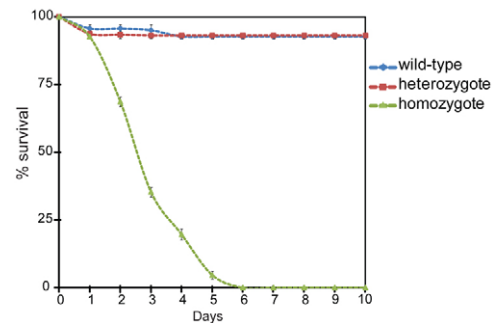


Fig. 5. *Pex1^{s4868}* homozygous flies have a reduced lifespan. Survival curve of *Pex1^{s4868}* homozygous (green), *Pex1^{s4868}* heterozygous (red) and wild-type (blue) flies. All *Pex1^{s4868}* homozygous flies died by day 6 (pupariation). Wild-type and heterozygous larvae pupated on day 6. Error bars indicate standard deviation.

Developing *Pex1* homozygous mutant embryos do not show obvious muscle defects

The locomotory defects observed in *Pex1^{s4868}* homozygous larvae (Fig. 6) could be attributed to aberrations in either neuronal or muscle development (or both). We therefore assayed the developing musculature in *Pex1^{s4868}* mutant larvae to determine whether the absence of *Pex1* affects muscle development in terms of gross morphology. Staining with an antibody to myosin (MAC147) showed an essentially wild-type pattern of musculature in the homozygous mutant embryos: both wild-type and homozygous mutant late (stage 15) embryos exhibited an evenly repeated pattern of longitudinal and oblique muscles (Fig. 7).

Pex1 homozygous mutants exhibit malformed central and peripheral nervous systems

Patients affected with a PBD present symptoms arising from significant defects in the CNS and peripheral nervous system (PNS), including mental retardation, seizures, muscular hypotonia and absence of deep tendon reflexes (Steinberg et al., 2006). We

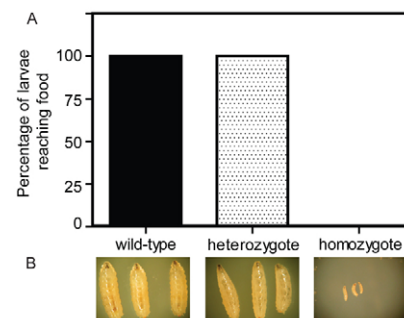


Fig. 6. *Pex1^{s4868}* homozygous larvae are smaller in size than wild-type and heterozygous larvae, and fail to show coordinated movement towards food. (A) A histogram reporting the percentage of larvae that reach food in a fixed period of 20 minutes. All wild-type and heterozygous larvae, but no homozygous larvae, were able to reach the food source in the prescribed time. (B) Images of 5-day-old wild-type, *Pex1^{s4868}* heterozygous and *Pex1^{s4868}* homozygous larvae. Homozygous larvae are much smaller than heterozygous or wild-type larvae.

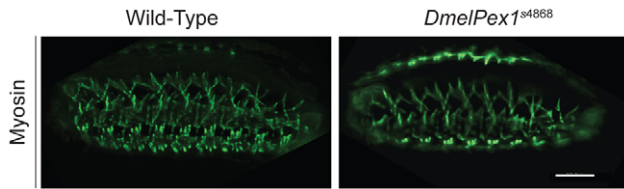


Fig. 7. *Pex1*^{s4868} homozygous embryos exhibit an essentially wild-type musculature. Wild-type and *Pex1*^{s4868} homozygous mutant embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibody MAC147 to myosin, which recognizes all muscle. Anterior is at right in all images. Lateral views are shown, dorsal is up. Scale bar: 100 μ m.

therefore examined the overall organization of the CNS and PNS in embryos homozygous for *Pex1*^{s4868}. Stage 15 embryos were stained with antibodies that decorate specifically the CNS and PNS, and were observed by immunofluorescence microscopy (Fig. 8). Monoclonal antibody BP102 stains axons of the CNS (anti-CNS axons) and is an excellent marker for the pattern of commissures and connectives in the CNS of embryos. Wild-type embryos decorated with BP102 showed a ventral nerve cord (VNC) with well-organized and well-formed anterior and posterior commissures and longitudinal connectives. By contrast, *Pex1*^{s4868} homozygous embryos showed malformation of the VNC, with a lack of some commissures, breaks in the longitudinal connectives and the presence of underdeveloped commissures, resulting overall in a widening of the distance between the longitudinal connectives of the VNC (Fig. 8).

Monoclonal antibody BP104 (anti-Neuroglian; Nrg) stains all neurons of the CNS and PNS and a small number of non-neuronal support cells in the PNS. We observed profound differences in staining between wild-type and homozygous mutant embryos when using anti-Nrg antibody (Fig. 8). In contrast to the CNS and PNS of wild-type embryos, *Pex1*^{s4868} homozygous embryos exhibited a marked loss and hypoplasia of neurons in the PNS, evidence of neuronal degeneration, disorganization of the neuronal pattern in the CNS and PNS, and severely malformed developing eye discs.

Staining with monoclonal antibody 22C10 (anti-Futsch) also showed dramatic differences in the CNS and PNS of wild-type and homozygous mutant embryos (Fig. 8). Wild-type embryos again exhibited a well-organized PNS and distinct neuron and/or neurite subsets within the VNC, whereas homozygous mutant embryos exhibited severe disruption, disorganization and loss of both PNS neurons and VNC neuron and/or neurite subsets.

Loss of *Pex1* causes disorganization in specific subsets of CNS and PNS neurons

We used monoclonal antibodies to Even-skipped (Eve; 2B8) and Fasciclin-2 (Fas2; 1D4) to probe more deeply the organization of the CNS and PNS in wild-type and *Pex1*^{s4868} homozygous mutant embryos (Fig. 9). Antibody to Eve stains the nuclei of a small subset of neurons in the CNS, whereas anti-Fas2 antibody recognizes the surface of a subset of neurons and axons in the VNC, including some motor neuron axons that innervate striated muscle cells in the periphery of the embryo. Eve expression in the CNS of a wild-type embryo is well organized and well defined when compared with that of the homozygous mutant embryo. However, the pattern

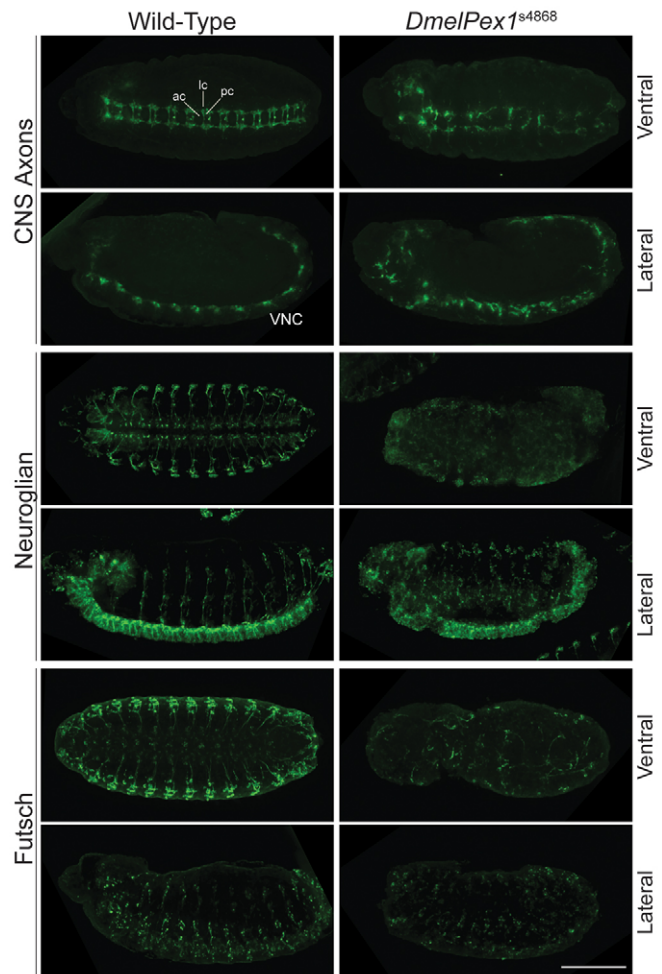


Fig. 8. The overall pattern of CNS and PNS development is abnormal in *Pex1*^{s4868} homozygous embryos. Wild-type and *Pex1*^{s4868} homozygous embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibodies BP102 (anti-CNS axons), BP104 (anti-Nrg; recognizing CNS and PNS neurons) and 22C10 (anti-Futsch; recognizing neuron and axon subsets of the CNS and PNS). Anterior is to the right in all images. In lateral views, dorsal is up for BP102 and 22C10 and down for BP104. ac, anterior commissure; lc, longitudinal connective; pc, posterior commissure; VNC, ventral nerve cord. Scale bar: 100 μ m.

of Eve expression in the anal plate is well organized in both wild-type and mutant embryos. Staining of homozygous embryos with anti-Fas2 antibody showed severe abnormalities in the VNC, extensive hypoplasia of developing eye discs and, in the brain, axon mislocalization and a reduction in the number of motor neurons and abnormalities in their organization (Fig. 9).

Anti-Cut antibody is known to demarcate a subset of cells in the PNS, specifically the nuclei of cells of external sensory organ precursors, including the Malpighian tubules, which are the *Drosophila* counterpart of the mammalian kidney. *Pex1*^{s4868} homozygous embryos exhibited massive abnormalities in the structure of developing Malpighian tubules and in the anterior and posterior spiracles, together with a severe loss of neurons in the CNS (Fig. 9).

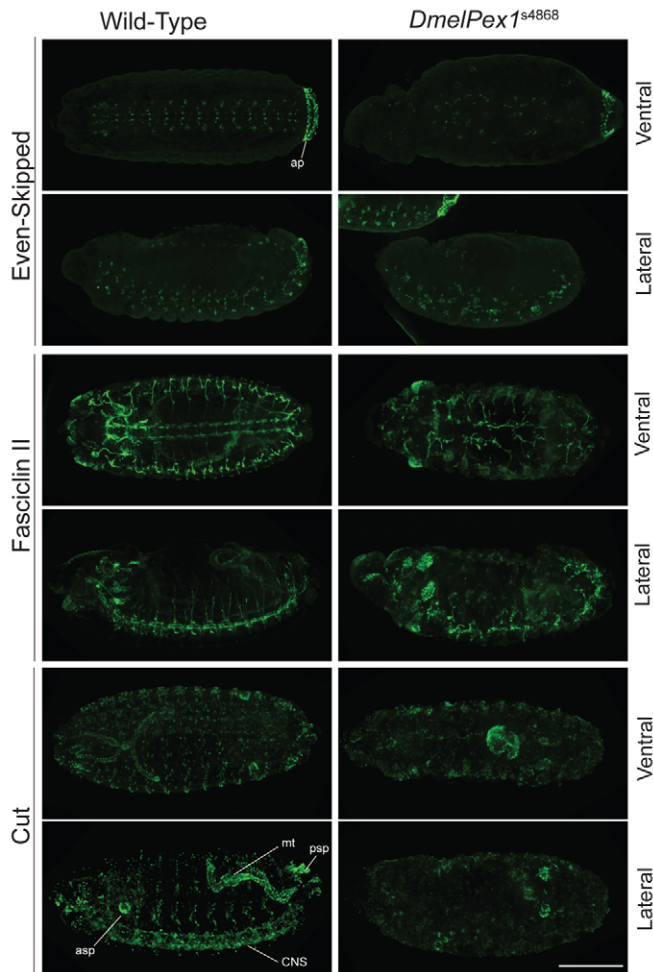


Fig. 9. CNS and PNS neurons are disorganized in *Pex1*^{s4868} homozygous embryos. Wild-type and *Pex1*^{s4868} homozygous embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibodies 2B8 (anti-Eve; recognizing the nuclei of a subset of CNS neurons), 1D4 (anti-Fas2; recognizing motor neurons and their axons in the ventral nerve cord) and 2B10 (anti-Cut; recognizing the nuclei of cells of external sensory organ precursors). Anterior is to the right in all images. In lateral views, dorsal is down for anti-Eve, and up for Fas2 and anti-Cut. ap, anal plate; asp, anterior spiracle; mt, Malpighian tubules; psp, posterior spiracle. Scale bar: 100 μ m.

***Pex1* homozygous mutant embryos also show disorganized glia**

Demyelination of axons occurs in the CNS of PBD patients (Steinberg et al., 2006). Although *Drosophila* lack myelin, glial cells perform a similar function in flies and humans (Freeman and Doherty, 2006). We used monoclonal antibodies to Reversed polarity (Repo; 8D12) and Wrapper (10D3) to label the glial cells of embryos (Fig. 10). Repo is a ubiquitous glial marker found in the nuclei of all glial subtypes and many PNS glia-support cells, but it is not expressed in the midline glia that ensheathe commissural axons. We used anti-Wrapper antibody to recognize midline glia. A dramatic disorganization of glia was observed in *Pex1*^{s4868} homozygous embryos compared with wild-type embryos. Taken altogether, our data demonstrate an essential role for the *Pex1* gene in the development of the fly nervous system.

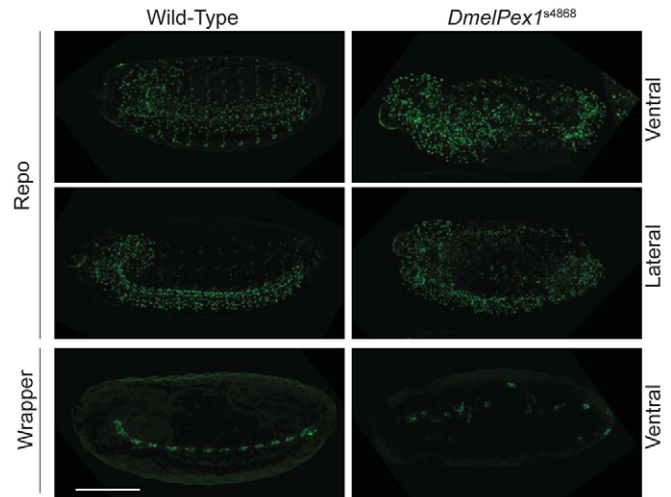
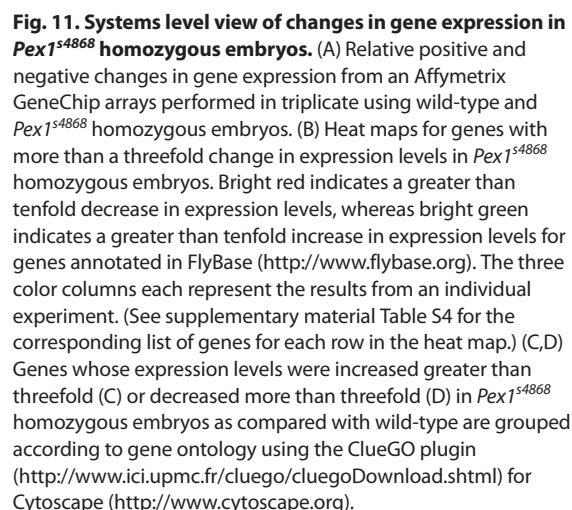


Fig. 10. Glial cells are disorganized in *Pex1*^{s4868} homozygous embryos. Wild-type and *Pex1*^{s4868} homozygous embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibodies 8D12 (anti-Repo; recognizing all glial cells except midline glia) and 10D3 (anti-Wrapper; recognizing midline glia). Anterior is to the right in all images. In lateral views, dorsal is up. Scale bar: 100 μ m.

Tissue defects correlate with genome-wide changes in gene expression in *Pex1* mutant embryos

Because so little is known regarding how mutations in the different PEX genes lead to the underlying pathology of PBD, we reasoned that an unbiased systems level approach would be a good starting point for elucidating new pathways and mechanisms. One strategy of potential use is a comprehensive expression analysis of transcription using microarrays. Loss of peroxisome function is expected to result in the dysregulation of metabolic pathways that could potentially lead to pan-genomic changes in gene expression at each of the cellular, tissue and organismal levels.

We performed a comparative genome-wide RNA expression analysis of transcription between wild-type and *Pex1*^{s4868} homozygous embryos (Fig. 11). Microarray analysis showed that the expression of 551 distinct protein coding genes showed a consistently greater than threefold difference in expression in 48-hour-old larvae homozygous for the *Pex1*^{s4868} mutation, compared with wild-type larvae (Fig. 11B). Of these genes, 396 were upregulated (supplementary material Tables S2, S4) and 145 were downregulated (supplementary material Tables S3, S5) in their expression vis-à-vis the wild-type condition. Notably, genes that were upregulated could be clustered by their predicted Gene Ontology (GO) annotations into several groups whose protein functions correlate strongly with the observed phenotypes of the *Pex1* homozygous mutant (Fig. 11C,D). These functions include neural development and activity; behavior; respiration; nucleotide biosynthesis; peptidase function, and adenylate cyclase and GTPase activities; and cell death. Additional pathways whose gene expression was upregulated include those involved with innate immunity, metabolism of purines and mitotic regulation (Fig. 11C; supplementary material Table S2). Genes whose expression was strongly downregulated in the *Pex1* homozygous mutant included, as expected, *Pex1* itself, as well as genes coding for transcription



factors involved in gametogenesis and proteins involved in taste sensation, proteolysis and vesicular transport (Fig. 11D; supplementary material Table S3). To control for a potential bias of association based on GO annotation, we also clustered randomly generated lists of genes, which typically generated fewer and sparser networks around annotations distinct from those generated by our microarray data (data not shown). However, 'nucleus organization' was a common annotation that appeared in both the randomly generated lists and our list, which might indicate that this annotation is an over-represented GO term.

DISCUSSION

Peroxisome assembly in *Drosophila* is mechanistically similar to human peroxisome assembly

To make robust conclusions regarding the suitability of *Drosophila* as a model for PBDs, compared with other models, it was necessary to determine whether the overall gene pathways required for peroxisome biogenesis in *Drosophila* are largely conserved with those in humans. Our systematic RNAi knockdown of 14 *Drosophila* genes that are homologous to mammalian and/or yeast PEX genes in S2 cells confirmed that at least 13 are bona fide Pex genes. Homologs of these genes have been shown to be required for peroxisome assembly in organisms from yeast to human (reviewed in Platta and Erdmann, 2007; Schrader and Fahimi, 2008). Importantly, mutation in 12 of these genes causes PBD and makes up 12 of the 13 known complementation groups in the PBDs. The thirteenth complementation group belongs to mutations in *Pex26*, a gene that we and others were unable to find in *Drosophila* (Schlüter et al., 2006). *Pex26* is the docking site for the AAA-ATPases *Pex1* and *Pex6* in humans, which are necessary for the recycling of the matrix protein receptor *Pex5* (Matsumoto et al., 2003). However, this receptor is not conserved across eukaryotes, and other unrelated receptors have been characterized in yeast (Elgersma et al., 1997; Birschmann et al., 2003) and plants (Goto et al., 2011). Despite being unrelated, all three of these proteins function similarly. Therefore, *Drosophila* most probably possesses a receptor for *Pex1* and *Pex6* that remains to be discovered.

Cells from PBD patients typically demonstrate increased cytosolic localization of peroxisomal matrix proteins. RNAi of *Drosophila Pex2, Pex6, Pex13, Pex16* or *Pex19* dsRNA produced clear reductions in the amounts of the corresponding mRNAs in treated S2 cells (supplementary material Fig. S1), whereas RNAi of the other Pex genes produced a less robust, yet still demonstrable, reduction in the corresponding mRNAs. Despite this variation in the reduction of mRNA levels, knockdown of *Pex1, Pex2, Pex3, Pex6, Pex12, Pex13, Pex14* or *Pex16* reduced or eliminated the punctate structures that are characteristic of peroxisomes, and mislocalized a GFP-SKL reporter to the cytosol.

Although the knockdown of *Pex5* by RNAi was somewhat variable, cells consistently showed an exclusively cytosolic signal for the GFP-SKL reporter. *Pex5* thus probably encodes the PTS1 receptor in *Drosophila*. Conversely, *Pex7* functions as the receptor for proteins targeted to peroxisomes by PTS2, independently of the PTS1(SKL) targeting sequence. This makes the knockdown of *Pex7* somewhat difficult to interpret, because we would not expect a change in the characteristic punctate pattern produced by GFP-SKL. Likewise, the presence of a putative *PEX20* homolog in *Drosophila* would suggest that there are parallels in the peroxisomal protein-import pathways

of insects and fungi (Titorenko et al., 1998). Several species of yeast have been shown to rely on the interaction between *Pex7p* and a *Pex20*-like protein for PTS2 protein import, whereas mammalian cells import PTS2 proteins via an interaction between *Pex7* and a larger splice variant of *Pex5* known as *Pex5L* (Girzalsky et al., 2010).

Pex19 protein has been shown to be required for peroxisomal membrane formation and has been suggested to function as the receptor for peroxisomal membrane proteins and/or as a chaperone acting to stabilize membrane proteins at the peroxisomal membrane. Absence of *Pex19* in most organisms leads to an inability of cells to assemble peroxisomes and an absence of identifiable peroxisomal membrane structures (Hettema et al., 2000). Interestingly, although we could show a clear and reproducible reduction in the mRNA of the putative *Drosophila* homolog of *PEX19* in RNAi-treated S2 cells (supplementary material Fig. S1), this nevertheless did not prevent the assembly of peroxisomes but instead led to a reduction in the number and increase in the size of punctate structures containing GFP-SKL and partial mislocalization of GFP-SKL to the cytosol. These differences in peroxisomal phenotype between the cells of other organisms lacking *Pex19* protein and S2 cells subjected to RNAi for *Pex19* mRNA could arise for a variety of reasons: (1) *Pex19* might not be the true *Drosophila* homolog of *PEX19* in other organisms. However, the degree of sequence similarity between human and *Drosophila Pex19* (Fig. 1) makes this unlikely. (2) *Pex19* protein might have functions in peroxisome biogenesis that are different from those of *Pex19* protein in other organisms. Because of the predicted multivariate nature of functions attributed to *Pex19*, further study of *Pex19* might provide a better understanding of *Pex19* function. (3) *Drosophila* might have another protein whose function in peroxisome biogenesis and peroxisomal membrane formation is redundant to that of *Pex19* protein. The de novo synthesis of peroxisomes from the endoplasmic reticulum (ER) has been shown to rely on additional proteins, such as *Dsl1p, Sec39p* and *Sec20p*, in *Saccharomyces cerevisiae* (Perry et al., 2009), and essential secretory proteins might play a more prominent role in this process in *Drosophila*. Other proteins, such as the p24 family of proteins (Marelli et al., 2004; Otzen et al., 2006), might also affect this trafficking pathway. The *Drosophila* system remains an excellent model organism for the discovery of new secretory mechanisms (Bard et al., 2006). (4) RNAi reduction of *Pex19* was insufficient to give a loss-of-function phenotype. A similar phenotype has been described for the yeast *Yarrowia lipolytica*, in which deletion of the *PEX19* gene results in the accumulation of peroxisomal structures that are similar in morphology to wild-type peroxisomes but are defective in matrix protein import (Lambkin and Rachubinski, 2001). Together, these observations suggest that *Pex19* might participate in several aspects of peroxisome biogenesis and be tailored for one or more aspects in individual organisms (Mast et al., 2010; Ma et al., 2011).

PEX11 is conserved among eukaryotes, with the role of controlling peroxisome division and proliferation (Fagarasunu et al., 2007). A loss of *Pex11* protein produces cells with decreased numbers of enlarged peroxisomes (Erdmann and Blobel, 1995). *Drosophila Pex11* probably functions similarly to *PEX11* genes in other organisms, because RNAi to *PEX11* transcript resulted in S2 cells containing few and enlarged peroxisomes but were unaffected in their ability to import GFP-SKL into peroxisomes.

Drosophila Pex23 exhibits limited similarity to *Y. lipolytica* Pex23p. Nevertheless, the peroxisome phenotype of RNAi-treated S2 cells (Fig. 2) is consistent with a role for *Drosophila* Pex23 in peroxisome biogenesis. Interestingly, the region of highest similarity between *Drosophila* Pex23 and *Y. lipolytica* Pex23p is in the unique dysferlin motif that is common to all members of the Pex23 protein family. Dysferlin motifs are thought to aid proteins in binding to certain phosphoinositides such as phosphatidylinositol 4-phosphate. This feature is proposed to aid the Pex23 family of proteins in their function of controlling peroxisome number and size in different yeast species (Brown et al., 2000; Vizeacoumar et al., 2004; Yan et al., 2008). S2 cells treated by RNAi against the transcript of *Pex23* had increased numbers of small peroxisomes as compared with control S2 cells, and no mislocalization of GFP-SKL to the cytosol. Therefore, notwithstanding the evolutionary distance between flies and yeasts, *Drosophila* has apparently retained some PEX genes that have heretofore only been reported in yeasts and have maintained their involvement in peroxisome biogenesis.

Pex1 mutants mirror the early developmental defects associated with human PBDs

A recent study of flies harboring mutant Pex2 and Pex10 peroxins has demonstrated a requirement for peroxisomes in normal spermatogenesis and the control of the levels of very-long-chain fatty acids in adult *Drosophila* (Chen et al., 2010). However, it is difficult to ascertain whether gametogenesis is affected in PBD patients, because mutation of the 13 PEX genes involved in PBDs cause early developmental defects and usually death within 1 year (Shimozawa et al., 2005; Steinberg et al., 2006). Because inherited mutations of *PEX1* are the most common cause of ZS, and of PBDs in general (Reuber et al., 1997; Steinberg et al., 2006), we wanted to examine the effect of *Drosophila* *Pex1* mutations on early development. The organismal phenotype associated with loss of Pex1 protein is strikingly similar to the ZS phenotypic spectrum. *Pex1* mutant larvae were smaller in size than their wild-type counterparts, failed to show coordinated movement or effective feeding habits, and exhibited severe malformations of the nervous system, with loss and/or mislocalization of axons and neurons in both the CNS and PNS during embryonic development. There are additional parallels between the abnormalities exhibited by ZS patients and flies mutant in the *Pex1* gene. ZS patients have severely demyelinated axons in both the CNS and PNS (Powers et al., 1985; Steinberg et al., 2006). Although *Drosophila* axons are not myelinated, *Pex1* mutant flies have extensively disorganized glial cells, which provide support and insulation to the axons of the CNS and PNS of flies in a manner akin to myelination in mammals (Freeman and Doherty, 2006). *Pex1* mutants also exhibit malformed Malpighian tubules, the functions of which are similar to those of mammalian kidneys. ZS patients also show structural abnormalities in the kidney (Steinberg et al., 2006).

The use of a simple genetic model such as *Drosophila* allows the examination of the cellular and organismal response to loss of *Pex1* in terms of gene expression. Thus, for the first time, we were able to evaluate the results of the loss of *Pex1* at a systems level using transcriptomic profiling. In mutant animals, the primary systemic molecular response to loss of peroxisome function can be linked to changes in 551 of the 13,767 protein coding genes (approximately 4%). Furthermore, the majority of these genes can be functionally

grouped into relatively few genetic pathways. However, the diversity of genetic pathways altered by loss of peroxisome function underscores the widespread and varied responses seen in both the clinical and biochemical presentation of PBDs. Our transcriptomic data reveal large-scale alterations in the expression of genes involved in chitin metabolism and puparial adhesion. This might implicate peroxisomes in the metabolic pathways of complex polysaccharides such as chitin, or might reflect changes in the secretory pathway through dysregulation and loss of peroxisome function. It has recently been demonstrated in the yeast *Pichia pastoris* that non-conventional secretion of acyl-CoA binding protein requires proper peroxisome metabolism (Manjithaya et al., 2010). These findings underscore our present lack of knowledge regarding peroxisome biology, which is essential for better understanding the disease progression of PBDs. The increase in expression of genes involved in innate immunity and humoral responses might be due to a response to increased levels of unused peroxisomal metabolites. Plasma levels of the plasmalogen precursors di- and trihydroxycholestanic acid are increased in PBD patients, as is a C₂₉-dicarboxylic acid that is not typically found in the serum of normal individuals (Ferdinandusse et al., 2009). The accumulation of these compounds and long-chain fatty acids that are esterified to cholesterol (Kovacs et al., 2004; Kovacs et al., 2009) are thought to contribute to the pleiotropic defects exhibited by PBD patients (Mast et al., 2010). However, changes in the expression of genes that are involved in innate immunity might underlie a currently unexplored role for peroxisomes in immune responses. Recently, mammalian peroxisomes were shown to function as signaling platforms for antiviral innate immunity (Dixit et al., 2010). The upregulation of genes involved in the immune response in *Pex1* mutants suggests that peroxisomes might also function in modulating the immune response of *Drosophila*.

Our profiling of changes in gene expression in response to loss of peroxisome function provides a tractable method to monitor disease progression. It might also prove to be a suitable tool for screening novel therapeutic drug candidates and monitoring disease regression in response to novel therapies of PBDs. Current attempts at finding small molecules and diet regimens that might alleviate symptoms of PBDs require extensive and time-consuming biochemical verification (Zhang et al., 2010; Dranchak et al., 2010). This process could be aided by transcriptomic profiling as a way to identify treatments that return gene expression profiles back to normal levels.

On the basis of the multiple classes of defects observed in our RNAi analysis in S2 cells, we have confirmed that at least 13 of the 14 known *Drosophila* Pex genes are required for what are probably different aspects of peroxisome assembly. Moreover, we have shown that specific developmental abnormalities in the developing embryonic nervous system occur when the *Pex1* gene is mutated. Taken together, our findings make a compelling argument for the use of *Drosophila* as a valid and tractable model system with which to investigate the roles of peroxisomes and the effects of PBD on the growth, development and response to the environment of a multicellular organism.

METHODS

Cell culture

Drosophila S2 cells were grown at 25°C in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% heat-inactivated

fetal bovine serum, 50 U penicillin/ml and 50 µg streptomycin sulfate/ml (Invitrogen). S2 cells constitutively expressing the fluorescent peroxisomal chimeric protein GFP-SKL (Kural et al., 2005) were kindly provided by Ronald Vale (University of California, San Francisco, CA).

Fluorescence microscopy

Embryos were fixed as described (Hughes and Krause, 1999) and stained with the following antibodies at 1:100 dilution (obtained from the Developmental Studies Hybridoma Bank, University of Iowa, IA): mouse anti-CNS axons (BP102), mouse anti-Neuroglian (BP104), mouse anti-Fas2 (1D4), mouse anti-Repo (8D12) and mouse anti-Wrapper (10D3) (all developed by Corey Goodman, University of California, Berkeley, CA); mouse anti-Futsch (22C10; developed by Seymour Benzer, California Institute of Technology, Pasadena, CA); mouse anti-Even-skipped (2B8; developed by Kai Zinn, California Institute of Technology, Pasadena, CA); and mouse anti-Cut (2B10; developed by Gerald M. Rubin, University of California, Berkeley, CA). Rabbit anti-GFP (Invitrogen) and rat anti-myosin (Abcam) were used at 1:1000 dilution. Alexa-Fluor-488-, Alexa-Fluor-568-, Cy2- and Cy5-conjugated secondary antibodies (Invitrogen, Jackson ImmunoResearch) were used at 1:2000 dilution to recognize primary antibodies. To reduce the effects of spherical aberrations, embryos were optically cleared in methyl salicylate after preparation for immunofluorescence (MacDonald and Rubel, 2008). Images were obtained using a UPlanFl 20×/0.5 NA air objective on an IX81 inverted epifluorescence microscope (Olympus) equipped with a CoolSNAP HQ digital camera (Roper Scientific) and an X-Cite 120 PC fluorescent illumination system (EXFO Life Sciences).

Images of RNAi-treated S2 cells were acquired using a Plan-Apochromat 63×/1.4 NA oil DIC objective on an Axiovert 200 inverted microscope equipped with a LSM510 META confocal scanner (Carl Zeiss). GFP-SKL was excited with a 488-nm argon laser and its emission collected with a 505-nm long-pass filter.

Images were deconvolved using algorithms provided by Huygens Professional Software (Scientific Volume Imaging BV, The Netherlands). For deconvolution, 3D data sets were processed to remove noise and reassign blur by an iterative classic maximum likelihood estimation widefield algorithm and a confocal algorithm, respectively, and an experimentally derived point spread function. Transmission images of S2 cells were processed by applying a Gaussian filter in Huygens, and blue color was applied to the transmission images using Imaris software (Bitplane). Transmission images were processed to maximize the fluorescent signal while maintaining cell outlines. Imaris was then used to render the deconvolved 3D data set to the processed transmission image. Final figure assembly was performed in Adobe Photoshop, Adobe Illustrator or Adobe InDesign.

RNAi knockdown of Pex homologs and semi-quantitative PCR

Templates for dsRNAs targeting predicted Pex homologs were isolated from a template library (Foley and O'Farrell, 2004). A control template to make dsRNA that specifically targets *Dredd* was a gift from Edan Foley, University of Alberta, Canada. dsRNAs were synthesized using the T7 RiboMAX kit (Promega) using template-specific primers (Foley and O'Farrell, 2004).

The dsRNA treatment of cells was performed as follows: day 1: cells were split 1:2 with fresh SFX culture medium (Invitrogen).

Day 2: 2×10^5 cells in 500 µl of SFX culture medium were added to each well of a 24-well dish (BD Biosciences); 20 µg of dsRNA to a gene of interest were added to one well, followed by gentle horizontal mixing; plates were sealed with Parafilm M and incubated at 25°C for 96 hours (days 2-5). Day 6: the cells in each well were split 1:2, and an additional 20 µg of dsRNA were added; the plates were resealed with Parafilm M and incubated at 25°C for 48 hours (days 6-7). Day 8: 10% of the remaining volume of cells was transferred to LabTekII chambered coverslips (Nalge Nunc) for microscopic examination of peroxisomes; total RNA was extracted from the remaining cells using RNeasy (Qiagen) and quantified with a NanoVue spectrophotometer (GE Healthcare); 200 ng of total RNA were used for reverse transcription using Superscriptase II (Invitrogen) and oligo-dT as the anchor primer; 2 µl of the resulting cDNA and gene-specific primers were then used in a 50-µl PCR reaction; for semi-quantitative PCR, specific Pex gene primers and control primers for a mRNA encoding a ribosomal protein (RP49) were used; the number of PCR cycles for each set of gene-specific primers was tested to ensure that the resulting reaction was within the linear range of amplification; products were separated by electrophoresis on 1% agarose gels; band intensity was measured using ImageJ and normalized to the control RP49 signal.

Preparation of antibodies to Pex1 protein and immunoblotting

Antibodies that recognize Pex1 were raised against a fusion of maltose-binding protein and the N-terminal 200 amino acids of Pex1 in guinea-pig and tested for specificity by immunoblotting of an S2 cell lysate using enhanced chemiluminescence (GE Healthcare). The protocol for raising the antibody is essentially as described before (Eitzen et al., 1997).

Drosophila stocks

Oregon R (wild-type) and *Pex1* (CG6760) strains *l(3)70Da^{s4868}* and *l(3)70Da¹* were obtained from the Bloomington *Drosophila* Stock Center at Indiana University. Stocks were maintained on standard Bloomington medium at 25°C, and embryos were collected at 25°C on apple juice agar plates. To facilitate the identification of homozygous mutant embryos, lethal *Pex1* mutations were maintained over the *w⁻*; *Sb¹/TM3, P{ActGFP}JMR2, Ser¹* balancer. UASp-Pex1 was made by TOPO cloning of a PCR fragment corresponding to the open reading frame of *Pex1* into pENTR/D (Invitrogen). This was transferred using Clonase LR (Invitrogen) into the plasmid pPW (Terence Murphy), which was transformed into *w¹¹¹⁸* embryos by P-element recombination.

Larval survival, growth and mobility assays

Parallel cultures of Oregon R and *l(3)70Da^{s4868}* adult flies were maintained for 1 week in a standard 2 L population cage at 25°C. Flies were allowed to lay eggs for 1 hour on apple juice agar plates, and cohorts of 100 randomly selected embryos of each genotype were placed on fresh apple juice agar plates with yeast paste at the center of the plates. The lifespan of *l(3)70Da^{s4868}* homozygous mutants was determined by observing larvae daily from hatching until death. Larvae were fed and watered regularly and kept in a 25°C incubator. The number of survivors was recorded daily. All homozygous mutants were dead by day 6. However, wild-type and *l(3)70Da^{s4868}* heterozygous larvae molted normally at day 6 and

TRANSLATIONAL IMPACT

Clinical issue

Mutation of genes that are responsible for the biogenesis and maintenance of peroxisomes cause a spectrum of debilitating disorders known as peroxisome biogenesis disorders (PBDs). Individuals with PBDs exhibit an array of clinical characteristics including ichthyosis, facial dysmorphology, retinitis pigmentosa, cataracts, deafness, hepatic disease, adrenal insufficiency, renal cysts, chondrodysplasia punctata, hypotonia, cerebral dysgenesis, and various forms of developmental, mental and physical retardation. Variability in the presentation of defects across the spectrum of PBDs reflects the central role of peroxisome metabolism in human development. The underlying cause of pathophysiology of the PBDs remains poorly understood and would benefit from a disease model that recapitulates aspects of the patient phenotype, enables functional characterization of disease mechanisms and increases understanding of the contribution of peroxisomes to normal physiology.

Results

In this study, the authors investigate the use of *Drosophila melanogaster* as a model in which to study the pathophysiology of PBDs. An in silico interrogation of the *Drosophila* genome identifies 15 putative homologs (Pex genes) of known human and yeast PEX genes. RNAi-mediated knockdown confirms that 13 of these putative Pex genes are required for peroxisome assembly in *Drosophila* S2 cells. For example, the *Pex1* gene encodes an AAA-ATPase, which, when mutated in humans, causes the most severe PBD, Zellweger syndrome (ZS). The authors show that knockdown of *Pex1* in S2 cells results in mislocalization of a peroxisome-targeted chimeric protein consisting of GFP and the peroxisome-targeting signal Ser-Lys-Leu (GFP-SKL) 3' to the cytosol. Furthermore, analysis of flies carrying a mutant form of the *Pex1* gene shows that *Pex1* is required for normal fly development, as is its homolog required for normal human development. Furthermore, mutant flies exhibit abnormalities characteristic of ZS patients, including developmental delay, poor feeding, severe structural abnormalities in the peripheral and central nervous systems, and early death. Finally, microarray analysis defines several clusters of genes whose expression varies significantly between wild-type and *Pex1*-mutant larvae, and that implicate peroxisomal function as a requirement for normal neuronal development, innate immunity, lipid and protein metabolism, gamete formation, and meiosis.

Implications and future directions

These results demonstrate that *D. melanogaster* is a useful model for studying PBDs. The finding that, as in humans, developmental programs in *D. melanogaster* require functional peroxisomes is demonstrated by the gross scale malformations noted in the organization of the central and peripheral nervous systems of mutant flies. In addition, these results reveal that dysregulation of genes involved in innate immunity and mitosis has a previously unappreciated role in disease pathology. Approaches to therapy for PBDs have been hampered owing to the rarity of these diseases and because of the lack of a cost-effective disease model. Future studies can make use of Pex-mutant flies to carry out large-scale screens to identify small molecules that might alleviate or reduce the developmental and physiological abnormalities associated with PBDs, which have thus far not been possible. Classes of molecules that suppress PBD phenotypes in flies could be developed as potential new treatments for PBD patients.

hatched into adult flies by day 10. The survival assay was repeated three times with 100 larvae in each experimental group. To assay larval growth, cohorts of 20 larvae of each genotype that had hatched were selected randomly after 24 hours. Staged larvae were placed on fresh apple juice agar plates with yeast paste at the center of the plates, allowed to grow for an additional 72 hours at 25°C, then visualized with a DF PLAPO 1.2× PF objective on a SZX12 microscope (Olympus) equipped with a PC1015 camera (Canon) and digitally photographed every 24 hours after hatching. Larval

cross-sectional area was measured using ImageJ software. Plates were monitored until day 4. The growth assay was repeated three times with more than 20 larvae in each experimental group. To assay larval mobility, larvae of each genotype were placed at one end of an apple juice agar plate and allowed to crawl to yeast paste placed at the other end of the plate. The distance crawled in 10 minutes was measured. 5-day-old larvae of each genotype were digitally photographed with a SZX12 microscope as above. Images were assembled with ImageJ and Adobe Photoshop.

Microarray analysis

Heterozygous *l(3)70Da^{s4868}/TM3, P{ActGFP}JMR2, Ser¹* parents were allowed to lay embryos on apple juice agar plates for 30 minutes at 25°C. The plates were then supplemented with yeast paste, and the embryos were allowed to develop for 48 hours at 25°C and 70% humidity. Hatched larvae were then hand sorted under a SX61 fluorescence stereomicroscope (Olympus) to select homozygous *l(3)70Da^{s4868}* larvae, i.e. those lacking GFP. Care was taken to select only larvae judged clearly to be alive as evidenced by their movement. Corresponding wild-type siblings were isolated from *w¹¹¹⁸/TM3, P{ActGFP}JMR2, Ser¹* parents. Total mRNA from 200 mg samples was isolated using Trizol (Invitrogen) and purified using RNeasy. The resulting RNA was tested for quality with an Agilent 2100 Bioanalyzer, converted to labeled complementary RNA (cRNA) and hybridized to *Drosophila* 2' 3'-UTR microarrays (Affymetrix). These experiments were run in triplicate. The resulting array data were normalized using the MAS5 algorithm and Expression Console software (Affymetrix). Genes showing a reproducible threefold increase or decrease in expression relative to their expression in wild-type siblings in three independent experiments were clustered based on their functional Gene Ontology annotations and known biochemical pathways using the DAVID package (Huang et al., 2009). Clustering of related genes according to their functional Gene Ontology annotations was done using the Cluego plugin (Bindea et al., 2009) for Cytoscape (www.cytoscape.org/).

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

S.C.H., A.J.S. and R.A.R. designed the research, analyzed the data and wrote the paper. F.D.M., J.L. and M.K.V. performed the experiments and analyzed the data.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.007419/-/DC1>

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