University of Alberta

Characterization of homologs of peroxisome assembly genes in the fruitfly *Drosophila melanogaster*

by

Maninder Virk

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Cell Biology

Spring 2011
Edmonton, Alberta

Examining Committee

Richard Rachubinski, Cell Biology

Andrew Simmonds, Cell Biology

John Locke, Biological Sciences

ABSTRACT

Peroxisomes are single-membrane-bound ubiquitous organelles required for several important metabolic pathways like lipid metabolism and peroxide detoxification. They are indispensable for normal human development, because lack of metabolically functional peroxisomes causes fatal peroxisome biogenesis disorders (PBDs). In this study, 14 putative *Drosophila melanogaster* (*D. melanogaster*) homologs of known *PEX* genes were identified using a dsRNA interference screen in cultured *D. melanogaster* S2 cells. In humans, mutations of the *PEX1* gene are the most common cause of the PBD, Zellweger syndrome.

Further, detailed phenotypic characterization of the *D. melanogaster PEX1* homolog (*DmelPex1*) showed that it is required for complete larval development. *DmelPex1* mutant larvae exhibit abnormalities similar to those observed in Zellweger syndrome patients, including developmental delay, poor feeding, structural abnormalities in the peripheral and central nervous systems, and premature death. Overall, this study supports the use of *D. melanogaster* as an invaluable model for the PBDs.

ACKNOWLEDGEMENTS

I am extremely thankful and indebted to my supervisor Dr. Richard Rachubinski for his constant support and encouragement throughout my graduate studies. Without his guidance and assistance this work would not have been possible. I admire his passion and enthusiasm for research.

I am thankful to all the members of the Rachubinski lab for their help and insightful feedback on my research during my graduate studies. I would like to thank the following past and present members of the Rachubinski laboratory: Fred Mast, Andrei Fagarasanu, Jenny Chang, David Lancaster, Robert Tower, Barbara Knoblach, Richard Poirier, Hanna Kroliczak, Elena Savidov, Dwayne Weber, Ryan Perry, and Melissa Dobson.

I would like to thank my supervisory committee for their support and guidance during my graduate studies. I am incredibly thankful to Dr. Andrew Simmonds for his guidance and helpful discussion related to my project. I would like to express my sincere thankfulness to Dr. John Locke for his time and help.

I would like to thank the Department of Cell Biology, the Faculty of Medicine and Dentistry, the Faculty of Graduate Studies and Research, and the Graduate Students' Association for their support.

Finally, I want to thank my family members and friends for their support during my graduate studies. I am especially grateful to my parents, my sisters, my brother, and my husband for their constant support, love and encouragement during my studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

/+	Heterozygous
3D	three-dimensional
CG	complementation group
CNS	central nervous system
DmelPex#	*D. melanogaster* wild-type *PEX#* gene
DmelPex#p	*D. melanogaster PEX#* protein
dsRNAi	double-stranded RNA interference
ER	endoplasmic reticulum
Eve	even-skipped
Fas2	fasciclin 2
FLP	flippase recombination enzyme
FRT	flippase recognition target
g	grams
GFP	green fluorescent protein
GFP-SKL	fluorescent chimera of green fluorescent protein and peroxisome targeting signal 1, Ser-Lys-Leu
IRD	infantile refsum disease
kDa	kiloDalton
L1	first instar larval stage
L2	second instar larval stage
L3	third instar larval stage

l	litre
LOF	loss-of-function
NALD	neonatal adrenoleukodystrophy
Nrg	neuroglian
PAGE	polyacrylamide gel electrophoresis
PBD(s)	peroxisome biogenesis disorder(s)
PCR	polymerase chain reaction
PEX#	wild-type gene encoding Pex#p
PNS	peripheral nervous system
PTS1	carboxy-terminal peroxisome targeting signal 1 with the consensus motif - (S/C/A)(K/R/H)(L/M)
PTS2	amino-terminal peroxisome targeting signal 2 with the consensus motif - (R/K)(L/V/I)X_5(H/Q)(L/A)
RCDP	rhizomelic chondrodysplasia punctata
RT	real time
SDS	sodium dodecyl sulphate
SKL	peroxisome targeting signal 1, Ser-Lys-Leu
UAS	upstream activation sequence
UTR	untranslated region
VNC	ventral nerve cord
ZS	Zellweger syndrome
ZSS	Zellweger syndrome spectrum

CHAPTER 1: INTRODUCTION

1.1. Overview of peroxisomes

First characterized biochemically by Christian de Duve and his group, peroxisomes are single-membrane-bound organelles found in virtually all eukaryotes (de Duve and Baudhuin, 1966; van den Bosch et al., 1992). Peroxisomes contain enzymes that are involved in a great variety of important biochemical pathways in different cell types (Cooper, 2000). Morphologically, peroxisomes range in size from 0.1 μm to 1.5 μm (Tolbert and Essner, 1981). Their number, shape, size, and protein composition vary dramatically depending on the organism, cell type and/or environmental conditions (Titorenko and Rachubinski, 2001a). For example, in mammals, peroxisomes are particularly abundant in the liver, kidneys, and central nervous system (CNS), where they are predominantly found in the oligodendroglia (Hawkins et al., 2007).

In humans and other mammals, peroxisomes contain more than 50 different enzymes that are utilized for a variety of important cellular functions and oxidative reactions such as the breakdown of fatty acids by α- and β-oxidation, free radical detoxification, oxidation of D-amino acids and polyamines, and synthesis of bile acids, plasmalogens, ether phospholipids, cholesterol and polyunsaturated fatty acids (Lazarow and Fujiki, 1985; van den Bosch et al., 1992; Singh, 1996; Wanders and Waterham, 2006; Schrader and Fahimi, 2008).

1.2. Importance of peroxisomes

Peroxisomes are essential for normal development. Metabolic dysfunction of peroxisomes is associated with a number of diseases ranging from relatively mild single enzyme deficiencies to the severe biogenesis disorders. In humans, the indispensable role of peroxisomes is emphasized by the severity and lethality of numerous peroxisomal disorders (Subramani, 1997; Gould and Valle, 2000; Subramani et al., 2000). These disorders can be divided into two major groups - the peroxisome biogenesis disorders (PBDs) and the peroxisomal single enzyme disorders (Wanders et al., 1996; Powers and Moser, 1998; Wander and Tager, 1998; Wanders, 1999; Fujiki, 2000; Wanders et al., 2001).

The PBDs are a heterogeneous group of autosomal recessive and X-linked neurodegenerative diseases that are incompatible with life or normal development (Schlüter et al., 2006). The PBDs are divided into two types - the Zellweger Syndrome Spectrum (ZSS) and the Rhizomelic Chondrodysplasia Punctata (RCDP) spectrum (Steinberg et al., 2006). The ZSS of peroxisomal diseases is the major subset and represents a clinical continuum with Zellweger syndrome (ZS) being the most severe form, followed by milder forms such as neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD) (Weller et al., 2003). Zellweger syndrome is also known as cerebrohepatorenal syndrome and is fatal in the first two years of life.

The ZSS disorders are present globally with an occurrence of approximately one in 50,000 live births (Gould et al., 2001). Patients with ZSS have impeded growth, muscular hypotonia, cataracts, cardiac defects, dysmorphic features,

mental retardation and profound neurological abnormalities (de Graaf et al., 1999; Shimozawa et al., 2005; Furuki et al., 2006; Steinberg et al., 2006). Neuropathologies in ZSS are characterized by abnormalities in neuronal migration and differentiation, defects in the formation of plasmalogens, hypomyelination, and post-developmental neuronal degeneration (Aubourg, 2007; reviewed in Shimozawa, 2007). The other distinct form of PBD, RCDP type 1, is also clinically characterized by abnormal psychomotor development, mental retardation, and death in early infancy (Purdue et al., 1999). Modeling the PBDs in a multicellular organism such as *D. melanogaster* can be useful for understanding the molecular and biochemical pathway defects that cause neuronal abnormalities in PBD patients.

1.3. Biogenesis of Peroxisomes

In recent years, two strategies have been very useful in the elucidation of the mechanism of peroxisome biogenesis and the identification of genes defective in PBD patients. Pioneering studies to identify genes involved in peroxisome assembly (*PEXs*) were performed in the budding yeast, *Saccharomyces cerevisiae*. This yeast has proven to be a powerful model organism for understanding the fundamental principles of peroxisome biogenesis, because the peroxisome levels can be easily controlled depending on the carbon source in the growth medium. For example, cells grown in glucose medium have very few peroxisomes; however, these same cells can be made to produce significantly more peroxisomes by transferring them to medium containing a peroxisomally metabolized carbon source such as oleic acid (Erdmann et al., 1989). In part due

to this control of peroxisome levels, yeast research has yielded ~32 different *PEX* genes (Wanders and Waterham, 2004; Platta and Erdmann, 2007). Consequently, various genetic alignment softwares were used to scan the human gene databases to identify human *PEX* gene orthologs. The identified genes were further tested by functional complementation analysis in peroxisome-deficient human fibroblasts. The second strategy involves making use of mouse knock-out models.

Currently, 32 *PEX* genes have been identified that encode peroxins, or peroxisomal proteins that are involved in regulating peroxisome assembly/biogenesis, division, and inheritance (Platta and Erdmann, 2007). And 13 of these *PEX* genes when mutated lead to lethal PBDs, suggesting that these genes are important for peroxisome biogenesis. Currently, PBDs comprise 13 complementation groups with their identified *PEX* genes (reviewed in Shimozawa, 2007).

These discoveries strongly suggest that there are multiple steps involved in peroxisome biogenesis or assembly and each peroxin has a particular role to play during one or more steps of this process (Fig. 1). The three distinct steps of peroxisome biogenesis include formation and assembly of peroxisomal membranes, import of peroxisomal membrane and matrix proteins, and peroxisome proliferation and maintenance. Peroxins Pex3p, Pex16p and Pex19p are required during the initial stages of peroxisomal membrane formation and/or assembly during peroxisome biogenesis (Fig. 1). Deletion of these peroxins results in the absence of detectable peroxisomal structures. However, structures

resembling wild-type peroxisomes are still present in the yeast *Y. lipolytica* deleted for Pex19p (Lambkin and Rachubinski, 2001).

All known peroxins are encoded by nuclear genes, synthesised on cytosolic polysomes, and targeted to peroxisomes post-translationally. During peroxisome biogenesis, the import of peroxisomal matrix and membrane proteins into peroxisomes requires several steps. These steps include cargo protein recognition in the cytosol by a receptor, docking of the receptor-cargo complex at the peroxisomal membrane, translocation of the receptor-cargo complex across the membrane, release of the cargo into the peroxisomal lumen, and receptor recycling to the cytosol. Peroxisomal matrix proteins are targeted to the peroxisomes via two distinct and conserved peroxisome-targeting signal sequences, a PTS1 (a carboxy-terminal tri-peptide with the consensus motif - (S/C/A)(K/R/H)(L/M) (Subramani, 1993) or a PTS2 (an amino-terminal nonapeptide with the consensus motif - (R/K)(L/V/I)X_5(H/Q)(L/A) (Rachubinski and Subramani, 1995). The majority of peroxisomal matrix proteins are targeted by PTS1, and a small subset is targeted by PTS2. Pex5p and Pex7p are the shuttling receptors that recognize cytosolic peroxisomal matrix proteins containing a PTS1 or PTS2, respectively, and form a receptor-cargo complex (Fig. 1) (Subramani, 1993; Marzioch et al., 1994; Rachubinski and Subramani, 1995; Terlecky et al., 1995). The PTS1 receptor Pex5p has a different role in yeasts than in mammals. In yeasts, it is involved only in the import of PTS1 proteins, whereas in mammals, Pex5p is involved in both PTS1- and PTS2-protein import. However, for both yeasts and mammals, Pex7p is involved in only PTS2-

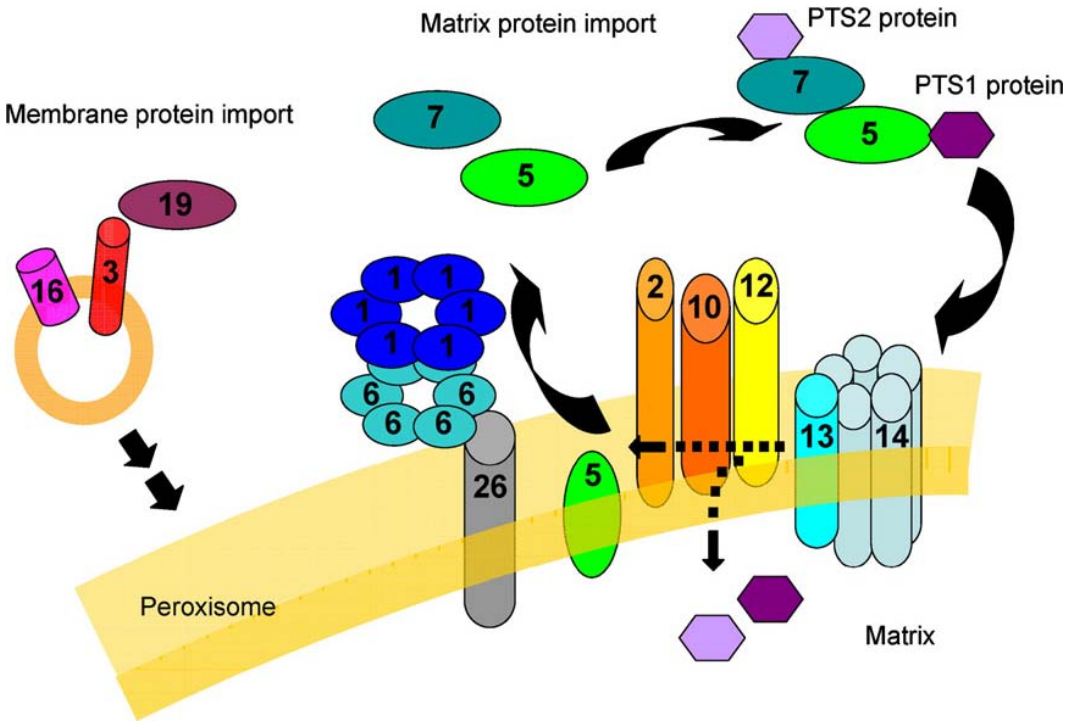

Figure 1. Model for peroxisomes biogenesis. The early peroxins PEX3, PEX16 and PEX19 are required during the initial stages of peroxisomal membrane formation and/or assembly. PEX5 and PEX7 are the shuttling receptors that recognise cytosolic peroxisomal matrix proteins containing a PTS1 or PTS2, respectively. The docking and translocation of receptor-cargo complexes at the peroxisomal membrane are facilitated by a docking complex (PEX13, PEX14 and PEX17) and a translocation complex (PEX2, PEX10 and PEX12). Peroxins implicated in the recycling of matrix protein receptors (PTS1 and PTS2) to the cytosol are PEX1, PEX6, and PEX26 (as taken form Steinberg et al., 2006).

protein import. The deletion of either Pex5p or Pex7p gives rise to cells that lack functional peroxisomes. On the other hand, peroxisomal membrane proteins (PMPs) are imported into peroxisomes by a distinct mechanism mediated by membrane PTS signals (mPTSs) (Hettema et al., 2000; Sacksteder and Gould, 2000).

The docking and translocation of receptor-cargo complexes at the peroxisomal membrane are facilitated by a docking complex (Pex13p, Pex14p and Pex17p) and a translocation complex (Pex2p, Pex10p and Pex12p). Peroxins implicated in the recycling of matrix protein receptors (PTS1 and PTS2) to the cytosol are Pex4p with its membrane anchor Pex22p and a complex containing Pex1p and Pex6p with its membrane anchor Pex15p (in *S. cerevisiae*) or Pex26p (in mammals) (Fig. 1). Malfunctioning or mutations of these recycling peroxins are most commonly associated with PBD-ZSS (Tamura et al., 1998; Platta et al., 2005; Furuki et al., 2006).

Peroxisome division and proliferation are controlled by peroxins belonging to the Pex11p and Pex23p families. Additionally, in all organisms, deletion of Pex11p leads to fewer and enlarged peroxisomes.

1.4. Functions of the *PEX1* gene

The *PEX1* gene encodes a 143-kDa cytoplasmic AAA-ATPase peroxin that is required for peroxisomal matrix protein import and is an essential gene that is required for complete peroxisomal biogenesis and/or assembly (Reuber et al., 1997). This gene is conserved from yeasts to human, and it plays an important role in the recycling of the peroxisomal matrix protein receptor Pex5p from the

peroxisomal membrane to the cytosol (Erdmann et al., 1991; Tamura et al., 1998; Platta et al., 2005; reviewed in Kiel et al., 2006). The human ortholog of yeast *PEX1* was identified by the groups of Reuber et al. (1997) and Portsteffen et al. (1997). Its mutation causes PBDs of complementation group 1 (Portsteffen et al., 1997). Peroxisomal protein import was restored upon expression of human *PEX1* in fibroblasts belonging to patients with PBDs of complementation group 1 (Reuber et al., 1997). Defects in the human *PEX1* gene are by far the most common cause of PBDs. Patients with two *PEX1* null alleles suffer from ZS and die within the first year (reviewed in Shimozawa, 2007).

1.5. Peroxisomes in the fruitfly, *D. melanogaster*

Research related to peroxisome biogenesis and maintenance has been extensively reported in several model systems like yeasts, *Caenorhabditis elegans*, and human cell lines (Lazarow and Fujiki, 1985; Titorenko and Rachubinski, 2001; Petriv et al., 2002). However, very little is known about peroxisomes in the metazoan, *D. melanogaster*. One early study reported the abundance of peroxisomes in the malpighian tubules and gut of adult *Drosophila* (Beard and Holtzman, 1987). Another study described the presence of a peroxide-generating oxidase in the peroxisomes of adult and larval *Drosophila* (St. Jules et al., 1989). In the following year, the same group published that peroxisomes are mainly localized in the head and are very abundant in the fat body of *Drosophila*. Their study implicated that the fat body lying adjacent to the eye is the main site for peroxisomal function. Furthermore, peroxisomes were involved in the processing of visual pigments, a process that occurs in the eye and malpighian

tubules of *Drosophila* (St Jules et al., 1990; Yagi and Ogawa, 1996; Southall et al., 2006). Recently, several studies reported the use of peroxisome-targeted chimeric proteins consisting of green fluorescent protein (GFP) and the peroxisome targeting signal Ser-Lys-Leu (GFP-SKL) to label peroxisomes in the *D. melanogaster* S2 cell line (Kural et al., 2005; Kim et al., 2007). A recent study reported the identification of 15 *Drosophila* peroxin genes and showed that peroxisomes are important for spermatogenesis in flies (Chen et al., 2010).

In this study we used *D. melanogaster* as a model organism to study the role of peroxisomes in *Drosophila* development.

1.6. The fruitfly, *D. melanogaster*, as a model system for the PBDs

In recent years, peroxisome-related research in single-cell systems like yeast and several mammalian cell lines has led to many breakthroughs and discoveries. However, knowledge about the role of peroxisomes in the development of multicellular organisms, PBDs, and evolutionary history is still incomplete (Schluter et al., 2006). Therefore, a broad approach and use of new simple model organisms to study peroxisomes is important to understand all aspects of this indispensible organelle, to define pathogenic mechanisms, and to evaluate new therapies.

Desirable traits of a disease model include small size, rapid generation time, high-throughput, low maintenance cost, ease of genetic manipulation, significant similarity to the human genome, well characterized anatomy, and availability of resources like gene and/or protein databases. The fruitfly, *D. melanogaster*, is one of the most studied and commonly used model organisms in

biology, including studies in genetics, development, and physiology. There are several advantages to using *D. melanogaster* as a model system: the flies are small, cheaply maintained, and easily reared in the laboratory. They have a short life cycle and high fecundity as compared to mice, making statistical analysis easy and reliable. At the same time, their genome is completely sequenced, with fifty percent of fly protein sequences having mammalian analogues. There are many techniques and protocols that can be easily employed to mutate/target/express specific genes in specific tissues.

Another major advantage of *D. melanogaster* is that its embryo grows outside the body and can easily be studied at every stage of development including embryogenesis, three larval stages (first instar, second instar, and third instar), a pupal stage, and the adult stage. The genotype of *D. melanogaster* is very simple and contains only four pairs of chromosomes; therefore, genetic manipulation of the fruitfly is very easy and feasible.

In a phylogenetic tree, *Drosophila* is more closely related to mammals than are yeasts or nematodes, and hence can provide closer links to human peroxisomes. At present, approximately seventy-five percent of known human disease genes have a recognizable match in the genetic code of *Drosophila* (Reiter et al., 2001), and various databases are in place to search for human disease gene homologues in flies and *vice versa*. *Drosophila* geneticists have successfully generated fruitfly models of more than 20 neurodegenerative diseases, including Huntington's disease, Parkinson's disease, Alzheimer's disease, fragile X mental retardation, and spinocerebellar ataxia 1 (Bilen and Bonini, 2005; Cauchi and van

den Heuvel, 2006; Raymond and Tarpey, 2006; Sanchez-Martinez et al., 2006).

1.7. Aim of this thesis

We wanted to establish *D. melanogaster* as a novel disease model for the PBDs. However, to use *D. melanogaster* as a potential disease model, we needed first to identify putative *PEX* genes in flies. For this we used RNA interference (RNAi), because it provides a direct link between gene sequence and functional data in the form of targeted loss-of-function (LOF) phenotypes (Echeverri and Perrimon, 2006). A systematic high-throughput dsRNA knockdown screen in cultured *D. melanogaster* S2 cells identified 15 putative fly *PEX* genes that are homologous to known *PEX* genes in other organisms. Another recent study reported the identification of some of the genes that are investigated in this study (Chen et al., 2010).

As mentioned previously, mutations in the human *PEX1* gene are the most common cause of one of the most severe types of PBD, Zellweger syndrome (Reuber et al., 1997; Steinberg et al., 2006). Interestingly, RNAi-mediated knockdown of the *D. melanogaster PEX1* homolog, *DmelPex1*, also known as *lethal (3)70Da*, (*l(3)70Da*), resulted in a failure to assemble peroxisomes and causes GFP-SKL mislocalization to the cytosol.

Further, we wanted to understand the functional role of peroxisomes in *D. melanogaster* development. For this purpose, we performed developmental studies and characterized flies homozygous for P-element insertions in *DmelPex1*, *l(3)70Da*, the homolog of the human *PEX1* gene. Based on the functional and encoded amino acid homology to human *PEX1*, *l(3)70Da* will be hereafter

referred to *as DmelPex1*. Homozygous *DmelPex1* mutants failed to develop normally past the larval stage and shared similar phenotypic symptoms as those observed in patients suffering from ZS. Overall, this study supports the use of *D. melanogaster* as a model system for studying the PBDs.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials used

2.1.1. Chemicals and reagents

Agarose	Invitrogen
Enhanced chemiluminescence	Amersham
Ethanol	Fisher
Fetal bovine serum	Invitrogen
Lanolin	Sigma-Aldrich
Methyl-salicylate	Sigma
Paraffin	Sigma
Paraformaldehyde	Biochemicals
Penicillin	Invitrogen
Potassium chloride (KCl)	BDH
Potassium phosphate, monobasic (KH_2PO_4)	EM Science
Sodium chloride (NaCl)	EM Science
Sodium dodecyl sulfate (SDS)	Bio-Rad
Sodium phosphate, dibasic (Na_2HPO_4)	BDH
Streptomycin sulfate	Invitrogen
Trizol	Invitrogen
Tween 20	Sigma-Aldrich
Skim milk	Carnation
Vaseline	Sigma-Aldrich

2.1.2. Molecular size standards

1kb DNA ladder (500-10,000 bp)	NEB
Prestained protein marker, broad range (6-175 kDa)	NEB

2.1.3. Multicomponent systems

Superscript Vilo cDNA synthesis kit	Invitrogen
T7 RiboMAX kit	Promega

2.1.4. Antibodies

Table 2-1. Primary antibodies

Name/Target	**Dilution**	**Source**
mouse anti-CNS axons (BP102)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-neuroglian (BP104)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-fasciclin 2 (1D4)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-repo (8D12)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-wrapper (10D3)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-futsch (22C10)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-even-skipped (2B8)	IF 1:100	Developmental Studies Hybridoma Bank
mouse anti-cut (2B10)	IF 1:100	Developmental Studies Hybridoma Bank

rabbit anti-GFP	IF 1:1,000	Invitrogen
rat anti-myosin	IF 1:1,000	Abcam
guinea pig anti-DmelPex1p	WB 1:1,000	This study

IF: immunofluorescence
WB: western blot

Table 2-2. Secondary antibodies

Conjugate	**Dilution**	**Source**
AlexaFluor- 488, 568	1:2,000	Invitrogen
Cy- 2, 5	1:2,000	Jackson ImmunoResearch Laboratories
Horseradish peroxidase (HRP)-conjugated anti-guinea pig IgG	1:30,000	Sigma-Aldrich

2.1.5. Oligonucleotides

Table 2-3. Oligonucleotides

Name	**Sequence**	**Application**
DmPex1_5′	CACCGAGATTGGGATCAATGCCAG	amplify specific regions in the 5′-UTR of *DmelPex1* mRNA
DmPex1_3′	CGCTCCATGTGATCCTGACGCTTG	amplify specific regions in the 5′-UTR of *DmelPex1* mRNA
Rpl32_5′	AGCATACAGGCCCAAGATCG	amplify specific regions in the 3′-UTR of *Rpl32* mRNA
Rpl32_3′	AGTAAACGCGGGTTCTGCAT	amplify specific regions in the 3′-UTR of *Rpl32* mRNA

2.2. *D. melanogaster* S2 cell culture

We used two different *D. melanogaster* S2 cell lines. One line constitutively expressed the green fluorescent peroxisomal chimeric protein, GFP-SKL (Kural et al., 2005).[1] The second line was the wild-type S2 cell line. These cells were cultured at 25°C in Schneider's *D. melanogaster* Medium supplemented with 10% heat-inactivated fetal bovine serum, 50 U penicillin/ml and 50 μg streptomycin sulfate/ml (Invitrogen).

2.3. dsRNA interference (RNAi)

To perform a dsRNAi-mediated knockdown of potential *DmelPex* homologs, we obtained templates from a *D. melanogaster* template library as previously described (Foley and O'Farrell, 2004). As a control for dsRNAi studies, we used a non-*PEX D. melanogaster* gene called *Dredd*.[2] dsRNAs were synthesized using the T7 RiboMAX kit (Promega) using template-specific primers (Foley and O'Farrell, 2004). dsRNAi experiments were performed in the following manner: Day 1, GFP-SKL S2 cells were plated into 24-well plates (BD Biosciences) at a density of 2×10^5 cells per well and a culture volume of 500 μl. dsRNA was then added to each well at a final concentration of 20 μg/ml, and cells were gently swirled a few times to let dsRNA mix with the plated cells. The plates were then incubated for 4 days at 25°C undisturbed. Day 4, cells were split 1:1 using Schneider's medium that was thawed at room temperature, and fresh dsRNA (20 μg/ml) was added to each well. Cells were incubated for an additional

[1] S2 cells constitutively expressing GFP-SKL were a kind gift from Dr. Ronald Vale, University of California, San Francisco.

[2] A control template to make dsRNA specifically against *Dredd* was a gift from Dr. Edan Foley, University of Alberta.

2 days at 25°C to allow for depletion of the corresponding gene product. Day 6, dsRNAi-treated cells were imaged.

2.4. *Drosophila* culture, strains, and embryo collection

D. melanogaster strains (Table 2-4) were maintained on standard Bloomington medium and kept in plastic vials at 25°C according to standard maintenance procedures. Vials were changed after every 7-10 days. Wild-type, heterozygous, and homozygous embryos were collected at 25°C from apple juice-agar plates at strict time intervals. This ensured that all embryos collected during a particular interval were at a similar stage of development. To facilitate the identification of homozygous mutant embryos, lethal *DmelPex1* mutations were maintained over the TM3-GFP third chromosome balancer.

Table 2-4. *D. melanogaster* strains

Strain Name	Genotype/description	Source
w1118 (Oregon R)	Carries a mutant eye color gene, otherwise wild-type; isogenic for chromosomes 1, 2 and 3.	Bloomington Drosophila Stock Center, stock #5905
Third chromosome GFP balancer	w^{-}; Sb^{1}/TM3, P{ActGFP}JMR2, Ser^{1}	Dr. Andrew Simmonds University of Alberta
P element insertion in CG6760	w^{1118}; P{lacW}$pex1^{s4868}$/TM3, P{ftz/lacC}SC1, ry^{RK} Sb^{1} Ser^{1}	Bloomington Drosophila Stock Center, stock#10177 ($l(3)70Da^{s4868}$)
X-ray mutagenesis in CG6760	$pex1^{1}$, mwh^{1} red^{1} e^{4}/TM3, P{Thb8-lacZ}WD1, Sb^{1} Ser^{1}	Bloomington Drosophila Stock Center, stock#4860 ($l(3)70Da^{1}$)
$l(3)70Da^{s4868}$/GFP	w+; $l(3)70Da^{s4868}$ / TM3, P{ActGFP}JMR2 this strain was used to characterize the *DmelPex1* mutant phenotype	This study
$l(3)70Da^{1}$/GFP	w+; $l(3)70Da^{1}$/ TM3, P{ActGFP}JMR2	This study

2.5. *D. melanogaster* embryo fixation

Wild-type and *DmelPex1* mutant embryos were collected on apple juice-agar plates (1.75% agar, 20% sugar, 50% (v/v) apple juice) at identical stages of development. They were fixed in 4% paraformaldehyde and then stained with several antibodies, as described previously (Hughes and Krause, 1999). To prevent any breakage or loss of embryos, they were treated very gently with no harsh mixing or shaking during the entire fixation and staining procedures. The fixed embryos were stored in methanol at -20°C.

2.6. Fluorescent staining of *D. melanogaster* embryos

For fluorescent antibody staining, fixed embryos were rehydrated in 2 ml of PBT (PBS [8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl] containing 0.1% Tween 20) for 15 minutes at room temperature. Rehydrated embryos were again washed with fresh PBT and then blocked for 1 hour in blocking solution (PBT + 0.5% powdered skim milk) to prevent non-specific antibody binding. After blocking, embryos were resuspended in 2 ml fresh blocking solution containing primary antibodies (described in Table 2-1) and placed at either room temperature for 90 minutes or overnight at 4°C with gentle rocking. Following this, embryos were washed twice for 5 minutes and four times for 15 minutes, using fresh blocking buffer each time. The washed embryos were then resuspended in fresh blocking solution containing secondary antibodies (described in Table 2-2) and incubated at room temperature for 2 hours in the dark with gentle rocking. Finally, embryos were washed twice for 5 minutes each and four times for 15 minutes each with fresh PBS.

2.7. Methyl salicylate clearing of *D. melanogaster* embryos

Fluorescently stained embryos were cleared in methyl-salicylate (Sigma). First, freshly stained embryos were washed twice with fresh PBS for 1 minute. Second, embryos were washed once with 50% ethanol for 5 minutes. Third, embryos were washed once with 70% ethanol for 5 minutes. Fourth, embryos were washed once with 90% ethanol for 5 minutes. Fifth, embryos were washed twice with 100% ethanol for 5 minutes, and after the second wash, all traces of liquid were removed from the tube. Sixth, 500 µl of methyl-salicylate was added to the tube very gently, and embryos were kept undisturbed for 10 minutes. Seventh, the methyl-salicylate was discarded, and replaced with 500 µl of fresh methyl-salicylate. After clearing, embryos were stored at 4°C.

2.8. Fluorescence microscopy

Cleared embryos were warmed to room temperature and then mounted on ordinary glass slides. To prevent the embryos from being crushed, vaseline was used to mark the boundaries of the slides before putting the coveslips on top. The edges of slides were sealed with Valap (1:1:1 mixture of vaseline, lanolin and paraffin). 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).

S2 cells were mounted on a glass slide and gently covered with coverslips before imaging. Images of the untreated, mock-treated and dsRNAi-treated GFP-SKL S2 cells were obtained with the help of a Plan-Apochromat 63×/1.4 NA oil

DIC objective on an Axiovert 200 inverted microscope equipped with an LSM510 META confocal scanner (Carl Zeiss). GFP-SKL was excited with the help of a 488-nm argon laser and its emission was collected with a 505-nm long-pass filter.

2.9. Deconvolution and image processing

Algorithms provided by the Huygens Professional Software (Scientific Volume Imaging BV, The Netherlands) were used to deconvolve the images of *D. melanogaster* embryos and dsRNAi-treated GFP-SKL S2 cells. To deconvolve the images, 3D data sets were first processed to remove noise and then reassign blur with an iterative Classic Maximum Likelihood Estimation, a widefield, and a confocal algorithm, respectively, and an experimentally derived point spread function. A Gaussian filter in Huygens was used to process the transmission images of S2 cells, and Imaris software (Bitplane) was used to apply blue color to these images. The levels of the transmission images were modified to maximize the fluorescent signal. However, the cell outlines were kept the same. Finally, Imaris was used to render the deconvolved 3D data set with the processed transmission image. Final figures of deconvolved embryos were assembled in Adobe Illustrator. Final figure of deconvolved S2 cells was assembled in Adobe InDesign.[3]

2.10. Preparation of antibodies to DmelPex1 protein and immunoblotting

Antibodies were raised in guinea pigs against the N-terminal 200 amino acids of DmelPex1p (performed by Elena Savidov, Department of Cell Biology, University of Alberta). S2 cell lysates were prepared for western blotting by

[3] Deconvolution and writing of Section 2.9 were done with the support of Fred D. Mast, University of Alberta.

adding SDS-PAGE buffer to cells, followed by immediate boiling for 5 minutes. After boiling, samples were centrifuged for a few seconds, and the supernatants were loaded onto SDS-PAGE gels. Immunoblotting was done using enhanced chemiluminescence (Amersham).

2.11. Purification of RNA from *D. melanogaster* S2 cells and larvae

Total RNA was extracted from S2 cells and lysates of the first instar larvae using Trizol reagent (Invitrogen) according to the manufacturer's instructions.

2.12. Development assay

Hundreds of wild-type Oregon R and *l(3)70Da*^{s4868} adult flies were collected for developmental assay. These flies were kept in standard 2-liter population cages for about one week in 25°C incubators. To collect embryos at a similar developmental stage, the initial 1-hour collection of eggs was always discarded. Following this, eggs laid by flies for an additional hour on apple juice-agar plates were collected and kept at 25°C for 24 hours. The next day, from each genotype, groups of 20 larvae that had hatched were randomly selected. Staged larvae were then placed on fresh apple juice-agar plates and allowed to grow for an additional 72 hours at 25°C. As a food source for these moulting larvae, yeast paste was regularly placed in the centre of the plates. Once the larvae hatched, their images were taken at the appropriate magnification every 24 hours using a DF PLAPO 1.2 × PF objective on a SZX12 microscope (Olympus) equipped with a PC1015 camera (Canon). At the same time, the larvae cross-sectional area was measured every 24 hours with the help of ImageJ software. All plates were monitored until Day 4.

2.13. Survival assay

Initially, hundreds of wild-type Oregon R and *l(3)70Da*^{s4868} adult flies were collected and kept in standard 2-litre population cages for about one week in a 25°C incubator. To collect embryos at a similar developmental stage, the initial 1-hour collection of eggs was always discarded. Following this, flies laid eggs for an additional hour on fresh apple juice-agar plates. From these plates, 100 embryos of each genotype were randomly selected and gently placed on fresh apple juice-agar plates. The embryos were placed in a 25°C incubator and allowed to hatch into first instar larvae. As a food source for the newly hatched larvae, yeast paste and water was regularly placed in the centre of the new plates. All larvae plates were kept in a 25°C incubator. For the survival assay, *l(3)70Da*^{s4868} homozygous mutant larvae were monitored daily from the day they hatched until they died. For each genotype, the numbers of surviving larvae were counted every day for the next few days. Some *l(3)70Da*^{s4868} homozygous mutant larvae died on Day 1. More than fifty percent of them were dead by Day 4, and all of them were dead by Day 6. In contrast, almost all wild-type and *l(3)70Da*^{s4868} heterozygous larvae exhibited normal moulting behaviour by Day 6 and hatched into adult flies within 10 days.

2.14. Crawling assay

Initially, hundreds of wild-type Oregon R and *l(3)70Da*^{s4868} adult flies were collected and kept in standard 2-litre population cages for about one week in a 25°C incubator. To collect embryos at the same development stage, the initial 1-hour collection of eggs was always discarded. Following this, flies laid eggs on

fresh apple juice-agar plates for a 2-hour period. Next, the plates were kept in a 25°C incubator for 24 hours so that the collected embryos could hatch into first instar larvae. On the next day, from each plate, several hundred larvae of each genotype were randomly selected and gently placed on fresh apple juice-agar plates. These larvae were allowed to grow for another 4 days at 25°C, and yeast paste with water was regularly placed in the centre of the plates. On day 5, larvae of each genotype were digitally photographed with a DF PLAPO 1.2 × PF objective on a SZX12 microscope (Olympus) equipped with a PC1015 camera (Canon). For the crawling assay, staged larvae of each genotype were individually placed at one end of the plate and were allowed to crawl to the yeast paste placed at other end of the plate. The distance traveled in a fixed amount of time by each larva was measured. Images were assembled in ImageJ and Adobe Photoshop.

2.15. Semi-quantitative RT-PCR

Trizol reagent (Invitrogen) was used to isolate total RNA from S2 cells and fly larvae according to the manufacturer's instructions.

A commercially available Superscript Vilo cDNA synthesis kit (Invitrogen) was used to perform reverse transcription. The experiment was performed in a 96-well plate (Eppendorf) according to the manufacturer's instructions, and 2.5 μg of total RNA were used for each reaction. Semi-quantitative PCR was done using 2.5 μl of a 1:20 dilution (*Rpl32*) or of a 1:5 dilution (*DmelPex1*) of the cDNA product. The primers used for the PCR reaction are described in Table 2-3. Specific regions in the 5′-UTR of *DmelPex1* mRNA and the 3′-UTR of mRNA encoding the *Rpl32* ribosomal protein were amplified

with these primers. *Rpl32* ribosomal protein was used as a loading control. Cycling conditions of the PCR reaction were 94°C for 3 minutes; 25 cycles of 94°C for 45 sec, 55°C for 30 sec, 72°C for 60 sec; 72°C for 10 minutes. A 2% agarose gel was used to separate the products of the PCR reaction, which were visualized by staining with ethidium bromide.[4]

[4] The experiment was performed by Dr. Andrew Simmonds, Section 2.15 is a report of his work.

CHAPTER 3: RESULTS

3.1. *In silico* approach to identifying putative *D. melanogaster* peroxins and peroxisomal enzymes

Putative *D. melanogaster PEX* (*DmelPex*) genes and peroxisomal enzymes were identified with an *in silico* screening and computational analysis of two main electronic *D. melanogaster* genomic databases at Information Hyperlinked over Proteins (iHOP) (http://www.ihop-net.org/UniPub/iHOP/) and the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Human peroxin and peroxisomal enzyme sequences were obtained from the NCBI. After an extensive search, 15 putative *DmelPex* genes were identified. Thirteen putative *DmelPex* genes were homologous to the known human *PEX* genes, and two were homologous to the yeast *Y. lipolytica PEX20 gene* and the yeast *S. cerevisiae PEX31* gene (Fig. 2).

Similarly, 10 putative *D. melanogaster* peroxisomal enzymes were identified as being homologous to known human peroxisomal enzymes (Fig. 2). Homologies were determined by using protein identity and/or similarity as the main selection criteria. The BLAST pairwise alignment tool was used to align homologous pairs of human peroxins and putative fly peroxins. Homologous pairs of human peroxisomal enzymes and putative fly peroxisomal enzymes were aligned in a similar manner.

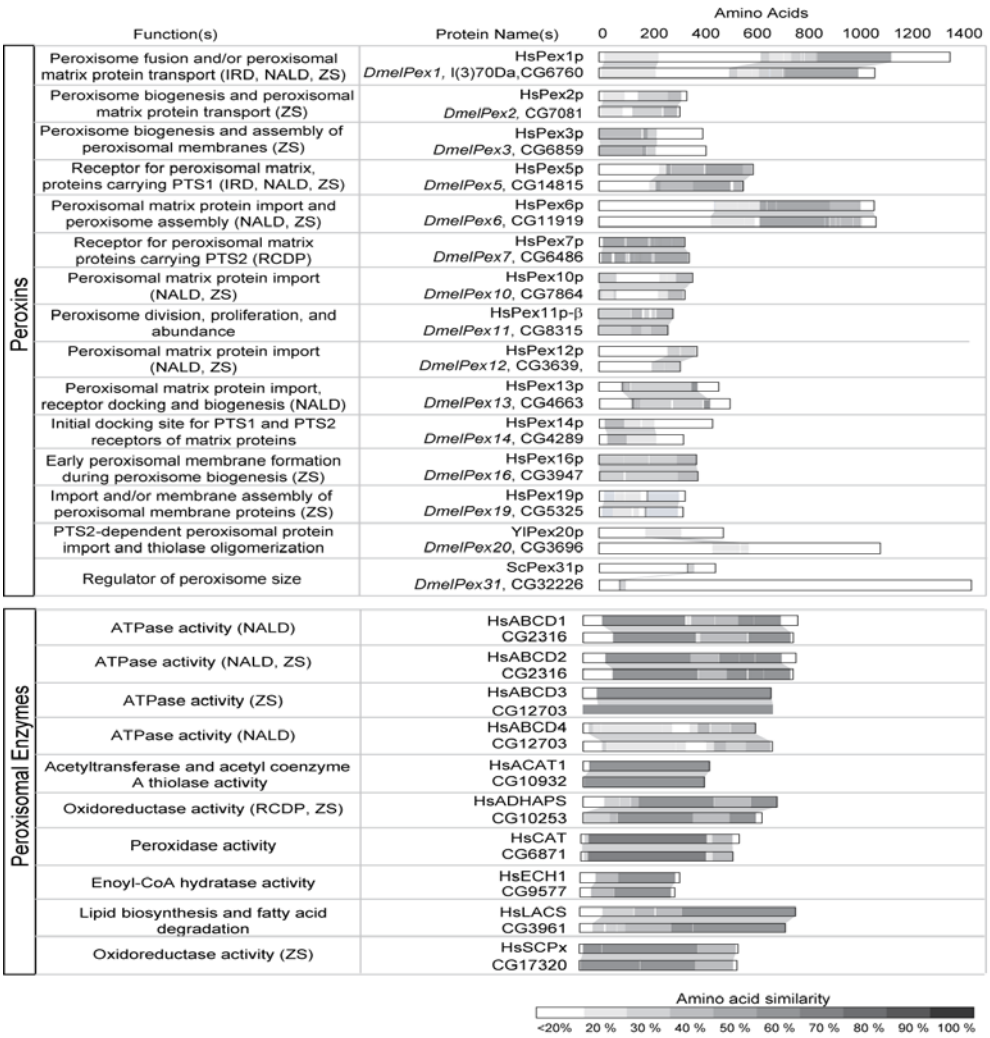

Figure 2. Peroxins and peroxisomal enzymes along with their putative homologs in *D. melanogaster*. The putative *D. melanogaster* homologs of known peroxins and human peroxisomal enzymes that were identified *in silico* are presented. The main function in peroxisome biogenesis of each known peroxin is given along with the main activities of the peroxisomal enzymes. The PBD in which a *PEX* gene and/or peroxisomal enzyme has been implicated is indicated in brackets. The SIM alignment algorithm (http://ca.expasy.org/tools/sim-prot.html) was used to make a pairwise alignment between the human or yeast peroxin or human peroxisomal enzyme (upper) and the putative *D. melanogaster* homolog (lower). The pairwise alignment is 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*; Sc, *Saccharomyces cerevisiae*; ABCD1, 2, 3 and 4, ATP-binding cassette subfamily D member 1, 2, 3 and 4, respectively; ACAT1, acetyl-CoA acetyltransferase 1; ADHAPS, alkyldihydroxyacetonephosphate synthase; CAT, catalase; ECH1, enoyl-CoA hydratase 1; LACS, acyl-CoA synthetase long-chain family member 1; SCPx, sterol carrier protein 2.

3.2. *DmelPex* genes are involved in peroxisome biogenesis

After identifying putative *DmelPex* genes, we wanted to find out if these genes had an effect on peroxisome biogenesis including the size, number, and/or morphology of peroxisomes. To do this, we performed a systematic dsRNAi-mediated knock-down of each identified gene in cultured *D. melanogaster* GFP-SKL S2 cell lines. These S2 cells constitutively express a green fluorescent protein (GFP) tagged at its carboxyl terminus with the PTS1 consisting of Ser-Lys-Leu (SKL) that specifically targets peroxisomes and produces a characteristic punctate pattern in fluorescence microscopy (Kural et al., 2005). Therefore, with the help of the peroxisome-targeted chimeric protein, GFP-SKL, we were able to visualize the effects of dsRNAi on peroxisomes in these cells.

GFP-SKL S2 cells were treated with double-stranded RNAs (dsRNAs) targeting 14 individual putative *DmelPex*s. Based upon the previously mentioned homology search, the following putative *DmelPex*s were selected for knock-down with dsRNAi: *DmelPex1* (*CG6760*), *DmelPex2* (*CG7081*), *DmelPex3* (*CG6859*), *DmelPex5* (*CG14815*), *DmelPex6* (*CG11919*), *DmelPex7* (*CG6486*), *DmelPex11* (*CG8315*), *DmelPex12* (*CG3639*), *DmelPex13* (*CG4663*), *DmelPex14* (*CG4289*), *DmelPex16* (*CG3947*), *DmelPex19* (*CG5325*), *DmelPex20* (*CG3696*) and *DmelPex31* (*CG32226*) (Fig. 2). For control experiments, we used a mock dsRNAi treatment (no dsRNA) and a dsRNAi-mediated knock-down of a non-*PEX* gene, *Dredd*.

The untreated GFP-SKL S2 cells had a punctate green fluorescence pattern characteristic of wild-type peroxisomes (Fig. 3) (Kural et al., 2005).

Similarly, in the case of mock-treated and *Dredd* dsRNAi-treated cells, the green fluorescence or GFP-SKL signal was present in punctate structures which were consistent with peroxisome localization in untreated cells (Fig. 3). Moreover, peroxisomes in both wild-type and control groups were spherical, of mostly uniform size, and randomly distributed throughout the cell. This shows that our control groups had wild-type peroxisomes and were not affected by non-specific dsRNAi (Fig. 3).

The dsRNAi-mediated knock-down of various putative *DmelPex* genes in GFP-SKL S2 cells resulted in different phenotypes (Fig. 3). These phenotypes were grouped into five main categories based on the pattern of GFP-SKL fluorescence obtained upon dsRNAi treatment as compared to the untreated or control-treated cells. In Category 1, the cells had a very diffuse cytosolic GFP-SKL fluorescence localization as compared to the untreated or control cells. This phenotype was observed when dsRNAi was done against the following putative *DmelPex* genes: *DmelPex1*, *DmelPex2*, *DmelPex5*, *DmelPex13* and *DmelPex16* (Fig. 3). In Category 2, cells exhibited a weaker cytosolic GFP-SKL fluorescence than cells in Category 1; however, the fluorescence was still localized to punctate structures representing peroxisomes. Also, in Category 2, the number of green punctate structures in cells was reduced compared to untreated or control cells. This phenotype was observed upon post-transcriptional silencing of the following putative *DmelPex* genes: *DmelPex3*, *DmelPex6*, *DmelPex12*, *DmelPex14* and *DmelPex19*. Interestingly, knock-down of the putative *DmelPex6* gene using dsRNAi against two separate regions of the gene (*DmelPex6 (i) and DmelPex6*

(ii)) gave rise to two different phenotypes. Cells treated with dsRNAi targeting *DmelPex6 (i)* did not exhibit any diffuse cytosolic localization of GFP-SKL and had an increased number of fluorescent punctate structures of variable size as compared to untreated or control groups. On the other hand, dsRNAi-mediated knockdown of *DmelPex6 (ii)* produced a Category 2 phenotype of a weaker cytosolic fluorescence and a reduced number of punctate structures (Fig. 3).

In Category 3 cells, a pattern of increased numbers of smaller fluorescent punctate structures but no increased cytosolic fluorescence was observed upon dsRNAi-mediated knockdown of the *DmelPex20* and *DmelPex31* genes (Fig. 3). In Category 4 cells, the GFP-SKL fluorescence pattern was similar to that of untreated cells. This category included the putative *DmelPex7* gene, and dsRNAi knock-down of this gene did not compromise the punctate fluorescence localization of GFP-SKL (Fig. 3). We anticipated this result, because Pex7p targets only those proteins to the peroxisomes that contain an amino-terminally located PTS2 and does not target proteins containing a carboxyl-terminally located PTS1 (Lazarow, 2006), like the chimeric reporter GFP-SKL being expressed in these S2 cells. Therefore, RNAi-mediated knock-down of this gene had no effect on GFP-SKL or PTS1 in GFP-SKL S2 cells.

Category 5 cells exhibited reduced numbers of enlarged fluorescent punctae punctae but no cytosolic fluorescence. This pattern was produced by dsRNAi knockdown of the putative *DmelPex11* gene (Fig. 3). In other organisms, the *PEX11* gene has been shown to function in the peroxisome division and/or

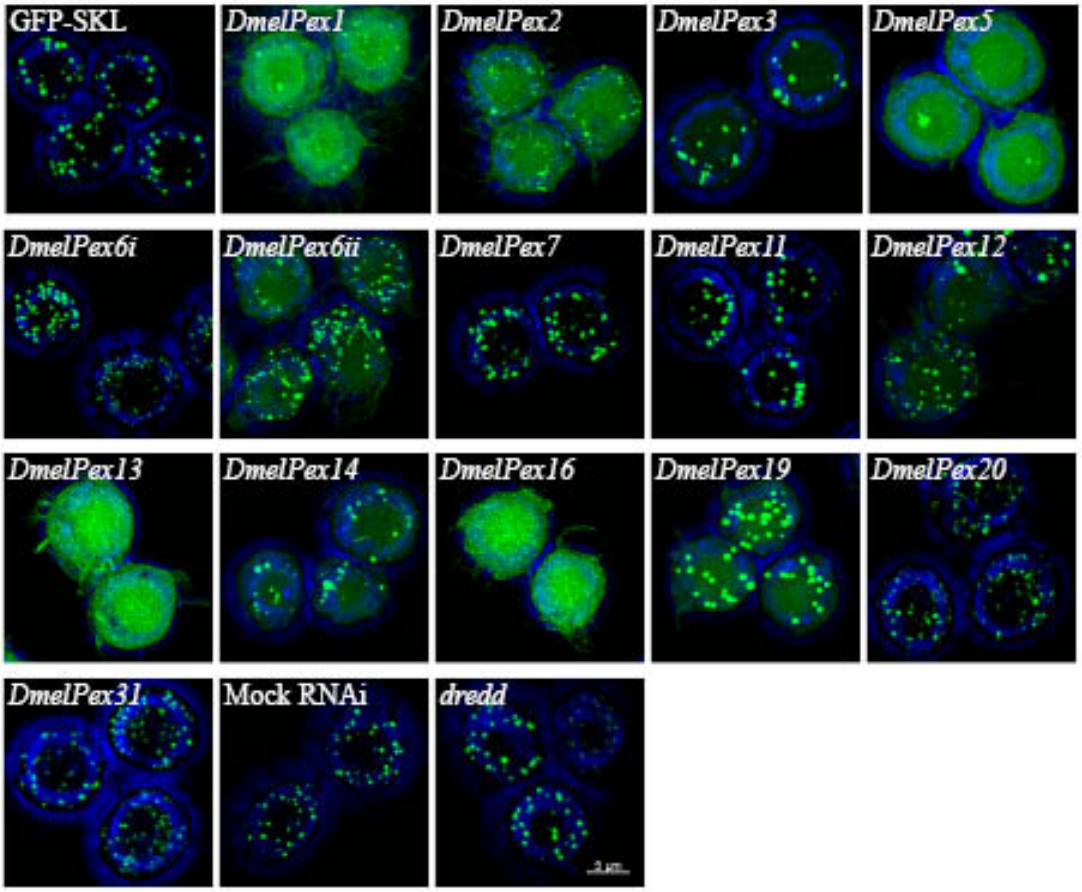

Figure 3. Peroxisomes are absent or exhibit altered morphology in S2 cells treated with dsRNA to putative *DmelPex* genes. S2 cells constitutively expressing the fluorescent peroxisomal reporter protein GFP-SKL (Kural et al., 2005) were treated with dsRNA to the indicated putative *DmelPex* genes, mock-treated, or treated with dsRNA targeting *Dredd*, a non-*PEX* gene. GFP-SKL in S2 cells targets to punctae characteristic of peroxisomes. Mock-treated cells and cells treated with dsRNA targeting *Dredd* exhibited punctae like control cells. Cells treated with dsRNAs to different *DmelPex*s exhibit mislocalization of the GFP-SKL peroxisomal reporter to the cytosol and/or altered peroxisomal size and number. Cells treated with dsRNA to *DmelPex7* exhibit punctate peroxisomes like those of wild-type cells, as *PEX7* affects the targeting only of peroxisomal proteins containing a PTS2 and not of those containing PTS1, such as GFP-SKL. Scale bar, 5µm.

proliferation process, and the absence of this gene produces a similar peroxisomal phenotype (Erdmann and Blobel, 1995; Fagarasanu et al., 2007).

Next, the transcript knockdown of the *DmelPex1* gene by dsRNAi-treatment was confirmed by doing a semi-quantitative RT-PCR (Fig. 4A) and by immunoblotting whole GFP-SKL S2 cell lysates with antibody to DmelPex1 protein (Fig. 4B). For the RT-PCR experiment, the expression levels of the gene *Rpl32* that encodes a ubiquitously expressed ribosomal protein was used as a loading control. RT-PCR results showed that the expression levels of *DmelPex1* mRNA were reduced in the S2 cells treated with *DmelPex1* dsRNA as compared to untreated, mock-treated, or cells treated with *Dredd* dsRNA (Fig. 4A). Immunoblot analysis showed that DmelPex1 protein was reduced specifically in lysates of S2 cells treated with *DmelPex1* dsRNA but not in the lysates of untreated cells, mock-treated cells or cells treated with dsRNA against the *DmelPex7* gene (Fig. 4B). In this experiment, a non-specific protein detected by the DmelPex1p antibody served as a loading control.

The initial dsRNAi screen in GFP-SKL S2 cells confirmed that we had successfully identified several putative fly *PEX* genes and that the majority of these genes functioned as *bona fide PEX* genes in *D. melanogaster* S2 cells (Fig. 3).

3.3. Abnormal embryonic development of the *DmelPex1* homozygous mutant flies was due to mutations in the *DmelPex1* gene

After studying these putative *DmelPex* genes (Fig. 2) in a single cell system, we wanted to look at them in a multi-cellular system using a whole

organism. dsRNAi-mediated knock-down of the majority of the identified putative *DmelPex* genes had an effect on peroxisome biogenesis in S2 cells. Therefore, we wanted to determine if compromising peroxisome biogenesis by mutating any of these putative *DmelPex* genes in living *D. melanogaster* would lead to defects in development. We started by specifically characterizing one of these *DmelPex* genes in the whole fly. We performed an in-depth phenotypic characterization of a mutation in the *DmelPex1* gene. We chose to focus our initial functional studies on the *DmelPex1*gene.

The *PEX1* gene is a good candidate for setting up *D. melanogaster* as a model system for studying PBDs development. In all organisms, the *PEX1* gene encodes a AAA- ATPase peroxin that is essential for peroxisome assembly (Reuber et al., 1997). Mutations in the human *PEX1* gene are the most common cause of the PBDs and account for at least 70% of patients with PBDs (Reuber et al., 1997; Steinberg et al., 2006). So far, many reports describe the mutational analysis of the *PEX1* gene and their effects on development (Maxwell et al., 1999; 2002; Walter et al., 2001; Poll-The et al., 2004). In humans, dysfunctional *PEX1* genes produce severe symptoms including neuronal defects, developmental delay, poor feeding, hepatic dysfunction, as well as musculoskeletal defects (Crane et al., 2005; Shimozawa, 2007; Fujiki et al., 2008). Patients with two *PEX1* null alleles have impaired development and generally die during the first year of life (as reviewed in Shimozawa, 2007). Because of the prevalence of the *PEX1* mutation in the PBDs, studies of *DmelPex1* mutations were a natural starting point for evaluation of *D. melanogaster* as a model system for the PBDs. Furthermore, the

DmelPex1 gene was already shown to be essential for peroxisome biogenesis in S2 cells (Fig. 3).

In this study we used two different types of *DmelPex1* mutant flies, one with a P-element insertion mutation (*l(3)70Da*^{s4868}) and the other with a X-ray-induced mutation (*l(3)70Da*^{1}) in the gene, respectively. Both of these mutations are lethal when homozygous. Since homozygous mutants were non-viable, heterozygous strains were used to breed homozygous mutants for studying their developmental defects. A GFP balancer placed over the mutant recessive lethal alleles *l(3)70Da*^{s4868} or *l(3)70Da*^{1} was used to distinguish between homozygous and heterozygous mutants. Balancers are recessively lethal, and embryos with two copies of the balancer chromosome die during embryogenesis. As a result, heterozygous mutant embryos (green) and homozygous mutant (non-green) embryos could easily be distinguished during development. Semi-quantitative RT-PCR showed greatly reduced levels of *DmelPex1* transcript in homozygous mutant *l(3)70Da*^{s4868} and *l(3)70Da*^{1} larvae 2 days after hatching (Fig. 4A). The mRNA isolated from 2 day-old wild-type larvae and *l(3)70Da*^{S4868} or *l(3)70Da*^{1} heterozygous larvae produced a specific band corresponding to *DmelPex1*. Whereas, *l(3)70Da*^{S4868} homozygotes produced no *DmelPex1* specific band, and *l(3)70Da*^{1} homozygous animals showed a severe reduction in this band compared to the *Rpl32* loading control (Fig. 4A).

The *D. melanogaster* life cycle is very short. It consists of an embryogenesis stage, three instar larval stages (first instar larva L1, second instar larva L2, and third instar larva L3), the pupal stage, and the adult stage. To look at

the developmental defects of *DmelPex1* mutant flies, I started with a developmental study that included growth, survival, and locomotory/crawling assays. *DmelPex1*^{s4868} homozygous mutants showed a reduced rate of hatching as compared to wild-type and heterozygous embryos (Fig. 5). The growth and survival assays showed that heterozygous *DmelPex1*^{s4868} flies grew (Fig. 6) and survived (Fig. 7) like wild-type flies. Both *DmelPex1*^{s4868} heterozygotes and wild-type flies developed into adult flies within 10 days. Similarly, in the crawling assay both wild-type and heterozygous *DmelPex1* larvae moved actively and reached the food source placed at one corner of the apple juice-agar plates within 20 minutes after hatching (Fig. 8). On the other hand, animals homozygous for the *l(3)70Da*^{s4868} allele had significantly reduced growth (Fig. 6) and died at the L1 or L2 stage (Fig. 7) as compared to wild-type and heterozygous larvae.

Also, *DmelPex1*^{s4868} homozygous larvae were much smaller in size and were unable to move actively or reach a food source when placed on agar plates (Fig. 8). These assays showed that *l(3)70Da*^{4868} homozygous larvae were smaller in size, had little or no locomotion, failed to show any effective feeding habits and exhibited developmental delay as compared to the wild-type or heterozygous animals. In some extreme cases, homozygous mutants were unable to crawl out of their eggshells and died within a few hours of hatching. It is important to note that these *DmelPex1*^{s4868} homozygous mutants exhibit incomplete penetrance. Overall, developmental studies showed that *DmelPex1* is important for normal embryonic development in flies.

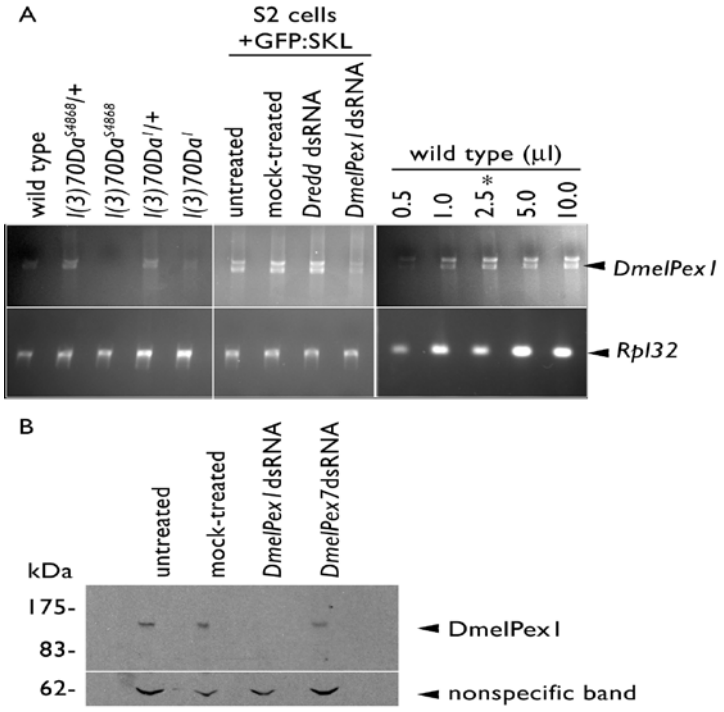

Figure 4. *DmelPex1* homozygous mutants and dsRNA-treated S2 cells have reduced levels of *DmelPex1* transcript, and S2 cells treated with *DmelPex1*dsRNA have reduced levels of *DmelPex1* protein. (A) The arrows point at specific bands corresponding to the expression levels of *DmelPex1* and the gene *Rpl32* encoding an ubiquitously expressed ribosomal protein which is used as a loading control. The symbol, /+ represents heterozygous *l(3)70Da* animals. The mRNA isolated from *l(3)70Da*^{S4868} homozygotes did not produce a *DmelPex1*-specific band, whereas a severe reduction in this band was seen in *l(3)70Da*^{1} homozygous animals as compared to the *Rpl32* loading control. Similarly, dsRNA-treated S2 cells had reduced levels of *DmelPex1* transcript as compared to untreated, mock-treated or S2 cells treated with a dsRNA that targets the *Dredd* gene, which is involved in the immune response. Specific amounts of the wild-type RT-reaction were analyzed for each set of primers and confirmed that 2.5 µl of experimental sample yielded a product within the linear range of amplification by the subsequent PCR.[5] (B) S2 cells treated with dsRNA to the *DmelPex1* transcript had reduced level of DmelPex1p protein. Lysates of untreated S2 cells, mock-treated S2 cells, S2 cells treated with dsRNA to *DmelPex1* mRNA and S2 cells treated with dsRNA to *DmelPex7* mRNA were separated by SDS-PAGE and subjected to immunoblotting with anti-DmelPex1 protein antibodies. DmelPex1 protein is reduced specifically in the lane containing lysate of S2 cells treated with dsRNA to *DmelPex1* mRNA. A protein detected nonspecifically by the antibodies to DmelPex1p is used as a control for protein loading. The migrations of molecular weight standards in kDa are represented by the numbers at left.

[5] This figure was assembled by Dr. Andrew Simmonds.

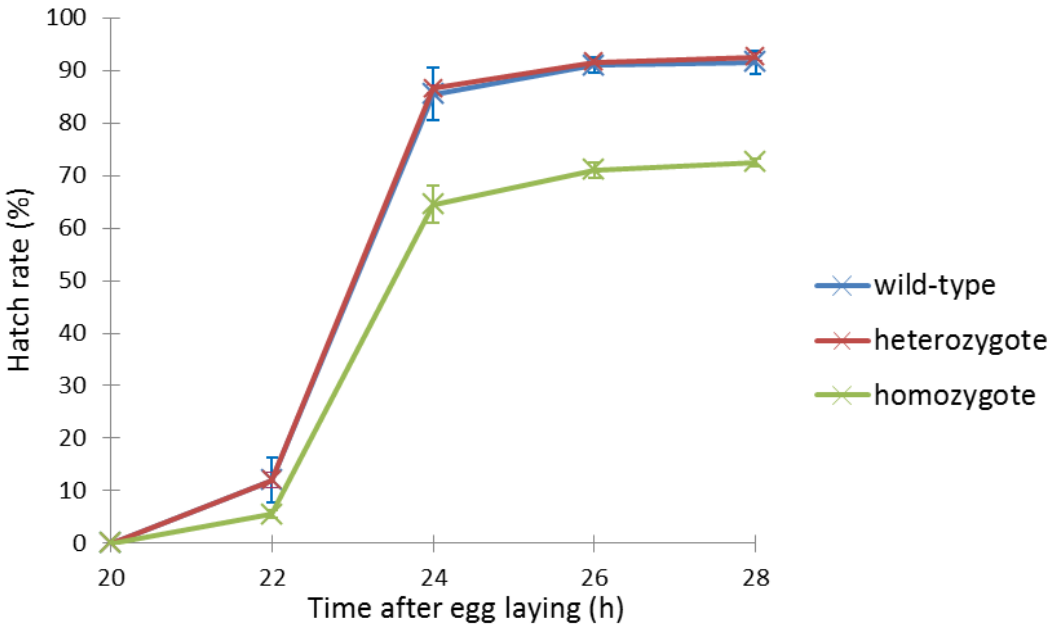

Figure 5. *DmelPex1*^{s4868} homozygous mutants display hatching defects. Hatching rate of *DmelPex1*^{s4868} homozygous (green), *DmelPex1*^{s4868} heterozygous (red) and wild-type (blue) embryos. *DmelPex1*^{s4868} homozygous mutants showed a reduced rate of hatching as compared to wild-type and heterozygous embryos. Error bars indicate standard deviation (n=2).

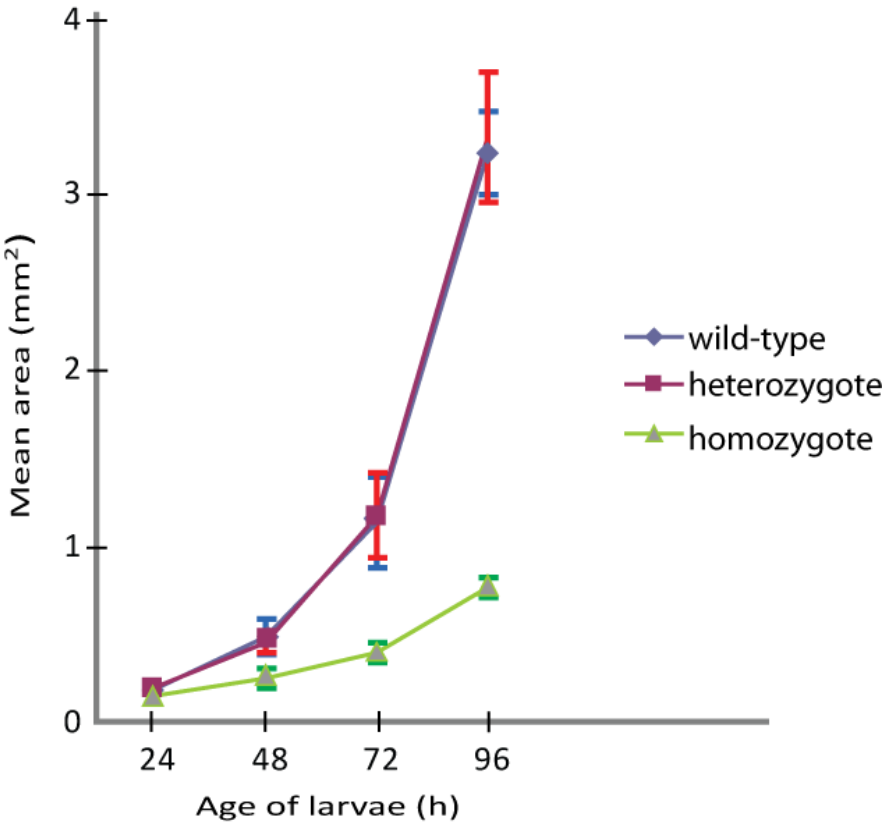

Figure 6. *DmelPex1*^{s4868} homozygous larvae exhibit growth defects. Each data point represents the average size (in mm^2) of 20 randomly selected *DmelPex1*^{s4868} homozygous (green), *DmelPex1*^{s4868} heterozygous (red) and wild-type (blue) larvae. On Day 4, the mean area representing growth of *DmelPex1*^{s4868} homozygous larvae is significantly reduced, as compared to 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 (n=3).

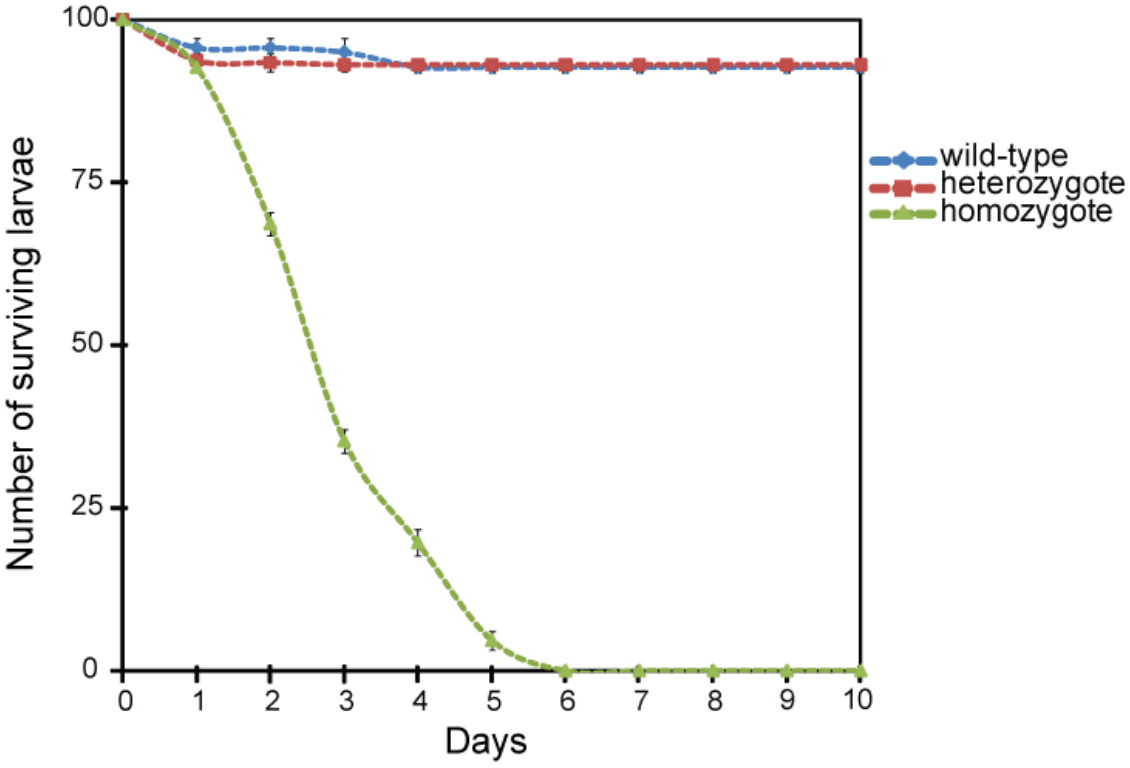

Figure 7. *DmelPex1*^{s4868} homozygous mutants do not survive past the larval stage. Survival curve of *DmelPex1*^{s4868} homozygous (green), *DmelPex1*^{s4868} heterozygous (red) and wild-type (blue) flies. All *DmelPex1*^{s4868} homozygous mutants are dead by Day 6 at pupariation. Wild-type and heterozygous larvae pupate on Day 6. Error bars indicate standard deviation (n=3).

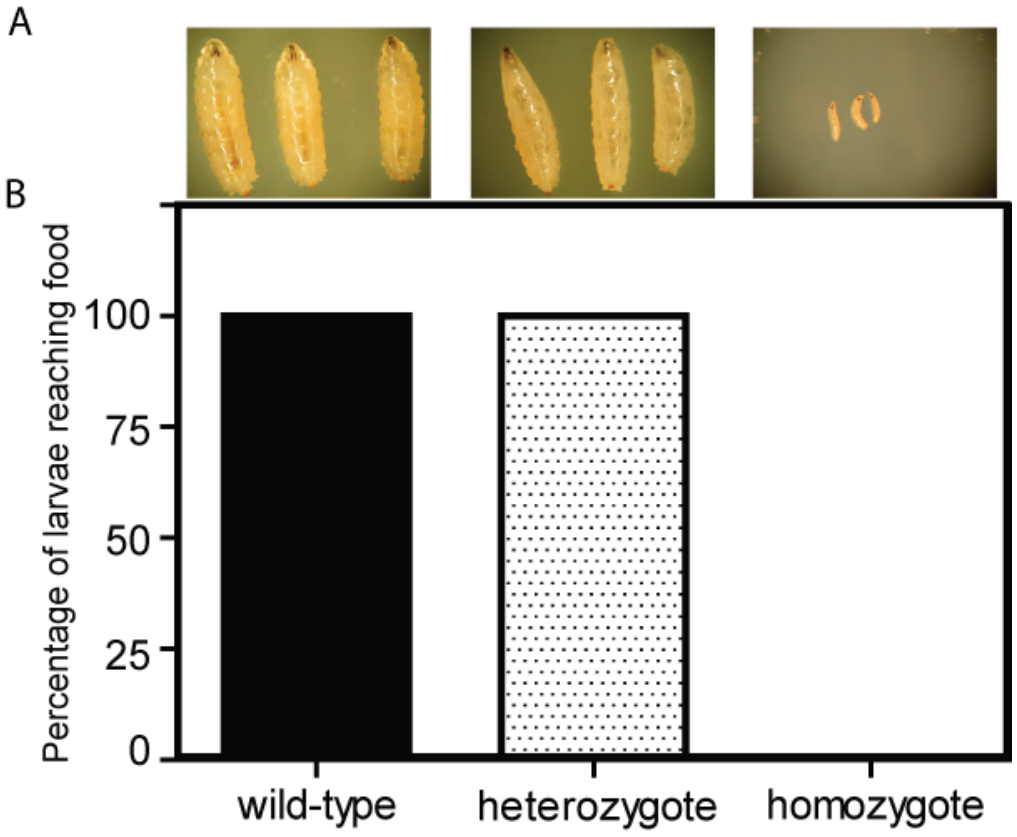

Figure 8. *DmelPex1*^{s4868} homozygous larvae are smaller in size and fail to move towards a food source. (A) Images of 5-day old wild-type, *DmelPex1*^{s4868} heterozygous and *DmelPex1*^{s4868} homozygous larvae. Homozygous larvae are much smaller in size as compared to heterozygous or wild-type larvae. (B) Percentage of larvae that reach food in a fixed period of 20 minutes. In the prescribed time, all wild-type and heterozygous larvae were able to reach the food; however, none of the homozygous larvae was able to reach the food (n=3).

3.4. *DmelPex1* homozygous mutants exhibit deformed central and peripheral nervous systems

As PBD patients suffer from defects in the central nervous system (CNS) and peripheral nervous system (PNS), we decided to investigate the development of these two systems in *DmelPex1*^{s4868} homozygous mutant embryos. 12-13-hour old (stage 15) embryos were stained with antibodies that specifically decorated their CNS and PNS. After, embryos were observed by immunofluorescence microscopy. The *DmelPex1*^{s4868} homozygous mutant animals show variable expressivity at different stages of development and we studied the affected mutants. The phenotypic variation is due to incomplete penetrance. Initially, we wanted to observe the nervous system of *DmelPex1*^{s4868} homozygous mutant embryos as compared to the wild-type embryos. For this, we used antibodies BP102, BP104 and 22C10 as these antibodies can stain a large portion of the CNS and PNS.

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. In wild-type embryos, the BP102 antibody decorated a ventral nerve cord (VNC). The VNC of wild-type embryos included well organized and well formed anterior and posterior commissures and longitudinal connectives. On the other hand, *DmelPex1*^{s4868} homozygous embryos showed a VNC that was severely malformed. The VNC of these embryos was missing some commissures and had breaks in the longitudinal connectives, along with the presence of underdeveloped commissures. Overall, these malformations resulted in a

widening of the distance between the longitudinal connectives of the VNC (Fig. 9).

Monoclonal antibody BP104 (anti-Neuroglian, Nrg) stains all neurons of the CNS and PNS, especially neurons that form neuron patterns in the PNS and developing eye disc. It also stains a small subset of non-neuronal support cells in the PNS. Profound differences between the wild-type and homozygous mutant embryos stained with anti-Nrg antibody were observed (Fig. 9). Wild-type embryos showed well formed and structured neurons in the CNS and PNS along with normal developing eye discs. *DmelPex1*^{s4868} homozygous embryos exhibited a marked loss of neurons in the PNS, hypoplasia of neurons in the PNS, disorganization of the neuronal pattern in the CNS and PNS, neuronal degeneration, and severely malformed developing eye discs.

Similarly, monoclonal antibody 22C10 (anti-Futsch) staining showed a dramatic difference between the CNS and PNS of wild-type and homozygous mutant embryos (Fig. 9). Anti-22C10 staining in wild-type embryos showed a well organized PNS along with distinct neuron and neurite subsets within the VNC. In contrast, homozygous mutant embryos exhibited severe disruption, disorganization and loss of both PNS neurons and VNC neuron and neurite subsets.

3.5. Specific disorganization in subsets of CNS and PNS neurons of *DmelPex1* homozygous mutants

After looking at the CNS and PNS neurons in wild-type and *DmelPex1*^{s4868} homozygous mutant embryos, monoclonal antibodies to Even-

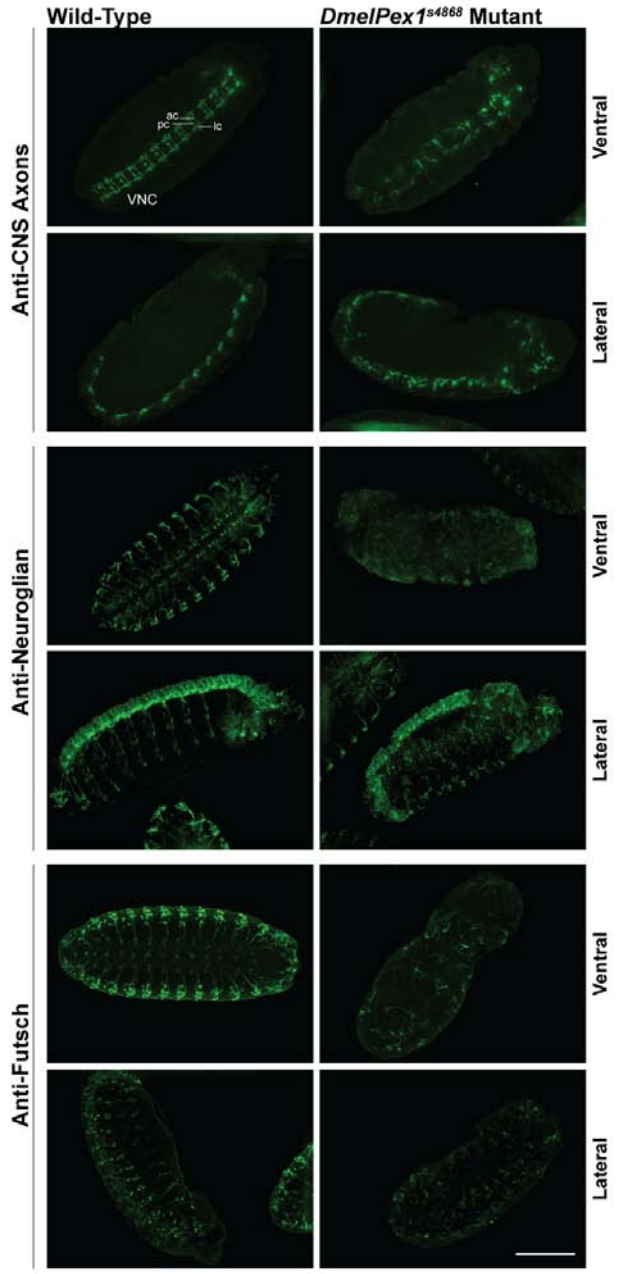

Figure 9. CNS and PNS development is abnormal in *DmelPex1*^{s4868} homozygous embryos. Wild-type and *DmelPex1*^{s4868} homozygous embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibodies BP102 (anti-CNS axons), BP104 (anti-Nrg recognizing CNS and PNS neurons) and 22C10 (anti-Futsch recognizing neuron and axon subsets of the CNS and PNS). Anterior is at 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 μm.

skipped (Eve, 2B8) and Fasciclin 2 (Fas2, 1D4) were used to probe more deeply the organization of the CNS and PNS (Fig. 10). Anti-Eve stains the nuclei of a small subset of neurons in the CNS, and anti-Fas2 antibody decorates 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 wild-type embryos was well organized and well defined as compared to the homozygous mutant embryos. However, the pattern of Eve expression in the anal plate of both wild-type and mutant embryos was well organized.

Homozygous mutant embryos stained with anti-Fas2 antibody exhibited severe abnormalities in the neurons and axons of the VNC, breaks in the VNC, extensive hypoplasia of the developing eye discs and in the brain region, and axon mislocalization. The number of motor neurons was also reduced in these embryos (Fig. 10).

Antibody 2B10 (anti-Cut) was used to stain a subset of cells in the PNS. This antibody specifically stains the nuclei of cells of external sensory organ precursors and the malpighian tubules. Malpighian tubules are the *D. melanogaster* counterpart of the mammalian kidney. *DmelPex1*^{s4868} homozygous embryos exhibited massive abnormalities in the structure of developing malpighian tubules and in the anterior and posterior spiracles, along with a severe loss of neurons in the CNS (Fig. 10).

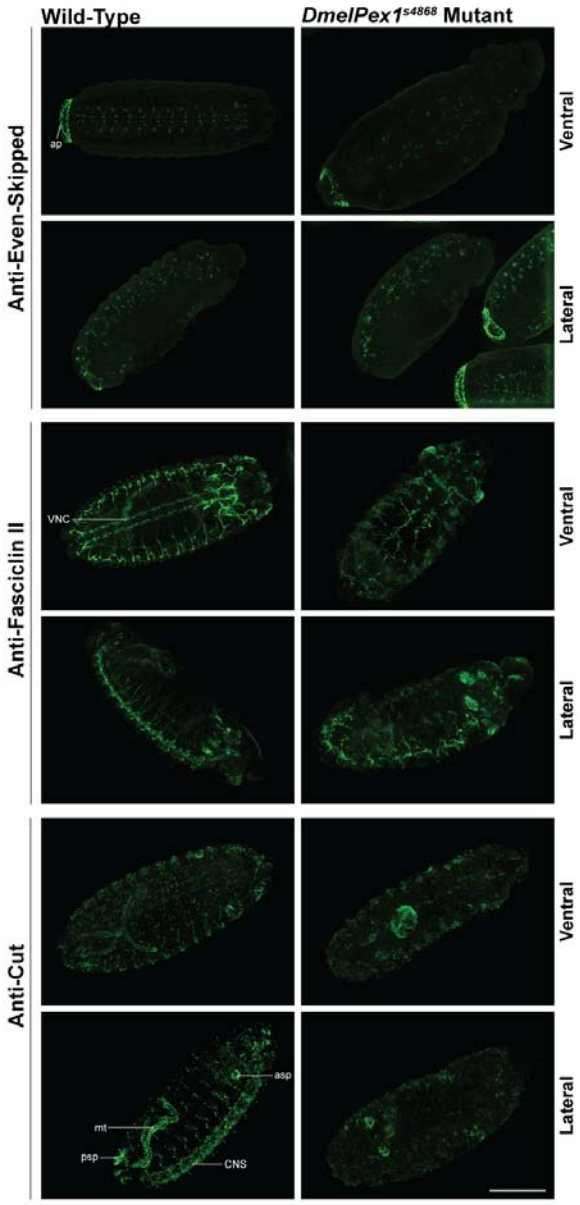

Figure 10. CNS and PNS neurons are disorganized in *DmelPex1*^{s4868} homozygous embryos. Wild-type and *DmelPex1*^{s4868} homozygous embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibodies 2B8 (anti-Eve recognizing the nuclei of a subset of CNS neurons), 1D4 (anti-Fas2 recognizing motor neurons and their axons in the VNC), and 2B10 (anti-Cut recognizing the nuclei of cells of external sensory organ precursors). Anterior is at right in all images. In lateral views, dorsal is down for anti-Eve and up for Fas 2 and anti-Cut. ap, anal plate; asp, anterior spiracle; CNS, central nervous system; mt, malpighian tubules; psp, posterior spiracle; VNC, ventral nerve cord. Scale bar, 100 μm.

3.6. *DmelPex1* homozygous mutant embryos exhibit disorganized glia

Patients with PBDs suffer from demyelination of axons in the CNS (Steinberg et al., 2006). Since *D. melanogaster* lacks myelin, glial cells perform a similar function in flies (Freeman and Doherty, 2006). The glial cells of wild-type and *DmelPex1*^{s4868} homozygous mutant embryos were examined by using monoclonal antibodies to Reversed Polarity (Repo, 8D12) and Wrapper (10D3) (Fig. 11). Repo is an ubiquitous glial marker that is expressed in the nuclei of all glial subtypes and many PNS glia-support cells. However, it is not expressed in the midline glia that ensheath commissural axons. Therefore, anti-wrapper antibody was used to stain midline glia. In contrast to the glia of wild-type embryos, *DmelPex1*^{s4868} homozygous mutant embryos exhibited a dramatic disorganization of glia when stained with both anti-Repo and anti-Wrapper antibodies (Fig. 11). Overall disruption of the CNS, PNS and glia in *DmelPex1*^{s4868} homozygous mutant animals suggests an essential role for the *DmelPex1* gene in the development of the fly nervous system.

3.7. *DmelPex1* homozygous mutant embryos have normal musculature

DmelPex1^{s4868} homozygous larvae did not move or crawl actively when placed on the apple-juice agar plates as compared to wild-type and heterozygous larvae. Also, these homozygous mutant larvae were unable to reach their food source (described above in Section 3.3, Fig. 8). Embryo musculature was examined to decide if absence of the *DmelPex1* gene affects muscle development in the homozygous mutants during the late embryonic stage. Muscles of the embryos were stained with a monoclonal antibody to myosin (MAC147). Both

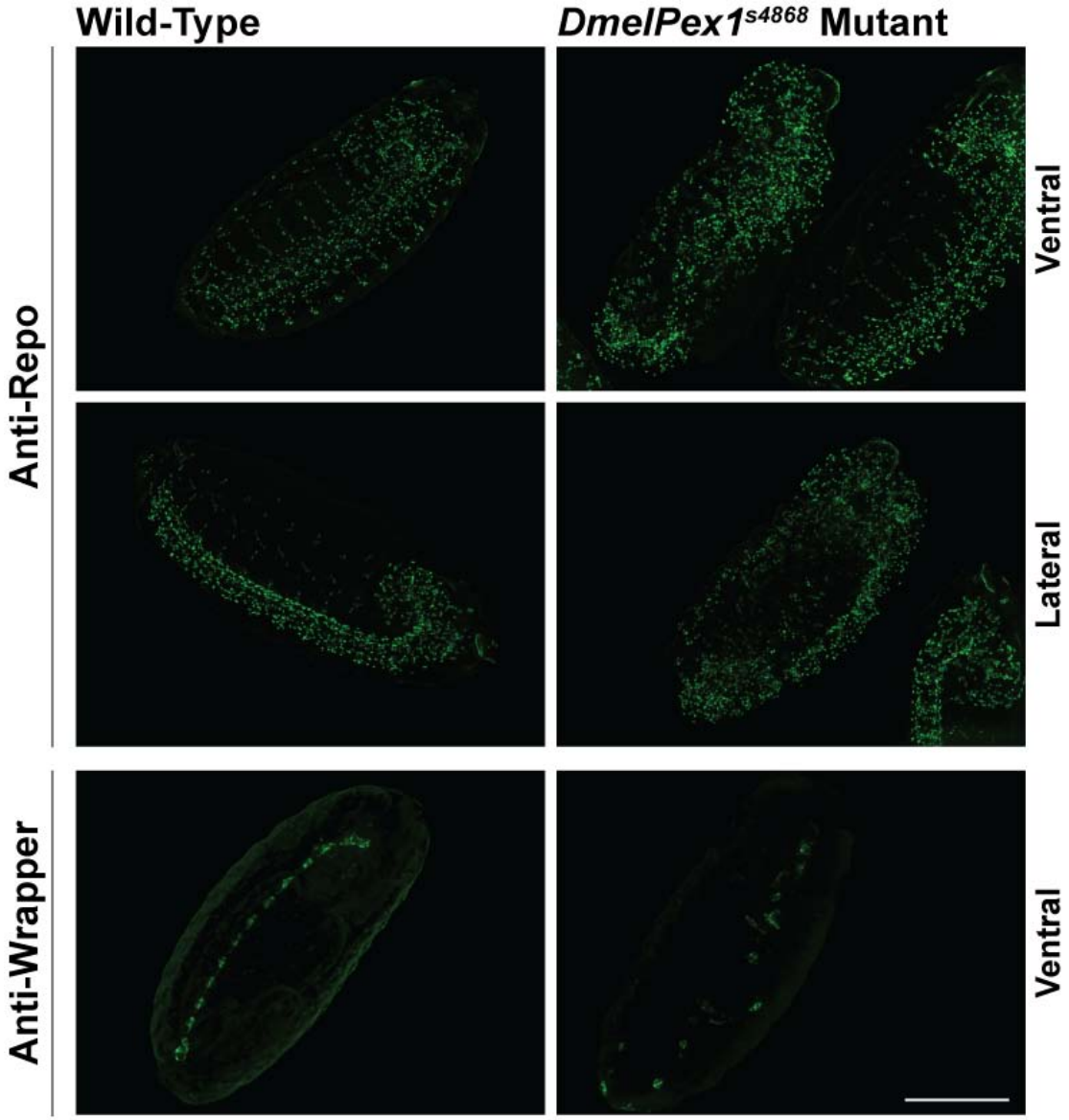

Figure 11. *DmelPex1*^{s4868} homozygous embryos exhibit disorganized glial cells. Wild-type and *DmelPex1*^{s4868} homozygous embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibodies 8D12 (anti-Repo recognizing all glial cells except midline glia) and 10D3 (anti-Wrapper recognizing midline glia). Anterior is at right in all images. In lateral views, dorsal is up. Scale bar, 100 μm.

wild-type and homozygous mutant embryos exhibited an evenly repeated pattern of longitudinal and oblique muscles (Fig. 12). Overall muscle development in the homozygous mutant embryos was not severely affected as they showed a wild-type pattern of musculature.

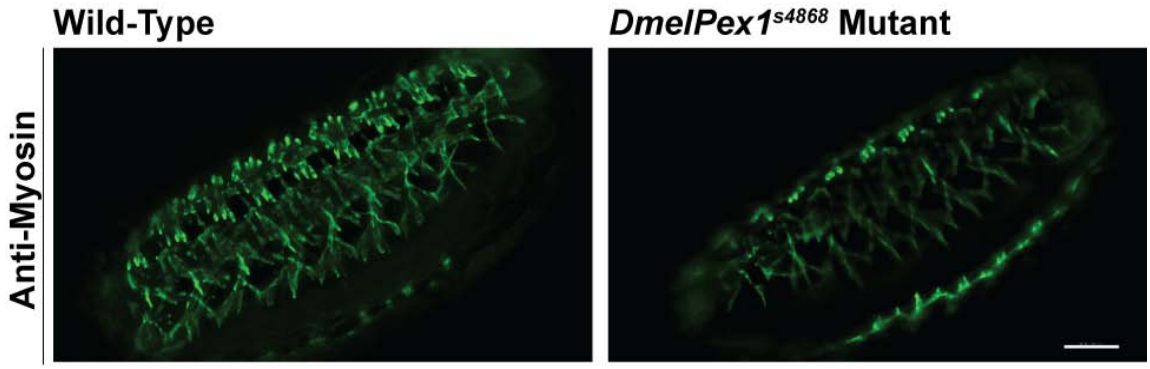

Figure 12. *DmelPex1*^{s4868} homozygous embryos exhibit a normal musculature. Wild-type and *DmelPex1*^{s4868} homozygous mutant embryos (stage 15) were analyzed by immunofluorescence microscopy using monoclonal antibody MAC147 (anti- myosin recognising all muscles). Anterior is at right in all images. In lateral views, dorsal is up. Scale bar, 100 μm.

CHAPTER 4: DISCUSSION

4.1. Characterization of *DmelPex* genes in *D. melanogaster*

RNAi is a technique where a specific gene product can be efficiently knocked-down by a dsRNA which has about 200 base pair homology to the gene of interest. Hence, RNAi provides information about the function of the gene in the form of loss-of-function phenotypes (Echeverri and Perrimon, 2006). One of the main advantages of using this technique is its success in cultures of *D. melanogaster* S2 cells. These cells are excellent for studying gene functions using the RNAi method because they are very sensitive to dsRNAi-mediated gene silencing, lack the interferon response, can efficiently take up dsRNA from the medium, and can easily be used for high-resolution microscopy (Clemens et al., 2000; Echeverri and Perrimon, 2006). Therefore, we used RNAi in cultured S2 cells to determine whether the identified 14 putative fly *PEX* (*DmelPex*) genes are involved in peroxisome function and/or biogenesis in *D. melanogaster*.

The S2 cell lines used in this study constitutively express a peroxisome-targeted green fluorescent chimeric protein between GFP and the carboxyl terminus peroxisome targeting signal Ser-Lys-Leu (GFP-SKL) (Kural et al., 2005; Kim et al., 2007). GFP-SKL S2 cells were treated with dsRNAs targeting 14 putative *DmelPex* genes and dsRNAi-mediated knockdown of some of these genes produced cytosolic localization of GFP-SKL and/or loss of green punctate structures representing peroxisomes. The cytosolic green fluorescent signal in the RNAi-treated groups could be due to the lack, or absence of, peroxisomes for

GFP-SKL targeting. Overall, RNAi results confirmed that 13 of the putative *DmelPex* genes are required for normal peroxisome assembly in S2 cells and function as *bona fide D. melanogaster Pex* genes (Fig. 13).

For RNAi studies we used dsRNAi-mediated knock-down of a non-*PEX* gene, *Dredd* as one of the control experiment. The *Dredd* gene was used because it is involved in the immunity pathway of flies and has no known role in peroxisome biogenesis or function. Interestingly, a recent study showed that peroxisomes have a role in innate immunity and that they are involved in promoting a rapid response to viral infections (Dixit et al., 2010). Therefore, for further studies a non-essential gene that is not involved in the immunity pathway of flies would serve as a better control. The dsRNAi-mediated knock-down of individual *DmelPex1*, *DmelPex2*, *DmelPex3*, *DmelPex6*, *DmelPex12*, *DmelPex13*, *DmelPex14* and *DmelPex16* genes in S2 cells led to mislocalization of the GFP-SKL signal to the cytosol and elimination or reduction of the fluorescently labeled punctate structures characteristic of peroxisomes. Interestingly, homologs of all these genes in organisms from yeasts to human have been shown to be required for the assembly of peroxisomes where they function at various steps of the peroxisome biogenesis process (for reviews, see Platta and Erdmann, 2007; Schrader and Fahimi, 2008).

Pex3p and Pex16p are required during the early steps of peroxisome biogenesis for the formation of the peroxisomal membrane and deletion of either of these two peroxins in many yeast species and human fibroblasts leads to the absence of peroxisomal membrane structures (ghosts) (Hettema et al., 2000;

reviewed in Schliebs and Kunau, 2004). RNAi against the *DmelPex3* gene resulted in S2 cells containing reduced numbers of fluorescently labeled peroxisomes as compared to control S2 cells. Interestingly, this suggests that in flies *DmelPex3* could have a role in peroxisome biogenesis. The *PEX16* gene has only been reported in humans and the yeast *Y. lipolytica*. This study shows the presence of the *PEX16* gene in flies. Human Pex16p is involved in peroxisome membrane assembly, and *Y. lypolytica* Pex16p is involved in regulating peroxisome proliferation (Eitzen et al., 1997; Kiel et al., 2006). RNAi studies in S2 cells imply that Pex16p of flies and humans has a similar function in the assembly and maintenance of peroxisomal membranes during early peroxisome biogenesis.

Pex5p acts as a receptor that recognizes matrix proteins containing PTS1 (SKL) and shuttles them from the cytosol to the peroxisome (Brown and Baker, 2003; Schell-Steven et al., 2005). As expected, dsRNAi-mediated knock-down of *DmelPex5* transcript produced a complete cytosolic signal for GFP-SKL, which contains the PTS1, Ser-Lys-Leu. The reduction in the level of the PTS1 receptor encoded by the *DmelPex5* gene affected the targeting of GFP-SKL to peroxisomes. On the other hand, dsRNAi against *DmelPex7* produced no cytosolic signal, and I did not observe any change in the characteristic punctate pattern produced by GFP-SKL. This is not surprising as Pex7p has been shown in a variety of organisms to function as the receptor for proteins targeted to peroxisomes by a PTS2 (Purdue and Lazarow, 2001; Brown and Baker, 2003). Therefore, reduction in the level of the PTS2 receptor encoded by the *DmelPex7*

gene did not affect the targeting of GFP-SKL to peroxisomes. Our preliminary RNAi results in S2 cells suggest that in flies *DmelPex5* and *DmelPex7* most likely encode the PTS1 and PTS2 receptor, respectively (Fig. 13).

Pex1p, Pex6p and Pex26p function in the recycling of the receptors that recognize matrix proteins from the peroxisome back to the cytosol following cargo release (Gould and Valle, 2000; Sacksteder and Gould, 2000; Fujiki et al., 2008). Studies of many organisms revealed that mutants for the *PEX1* and *PEX6* lack morphologically detectable peroxisomal structures and mislocalise peroxisomal matrix proteins to the cytosol (Erdmann et al., 1991; Van der Leij et al., 1992). Interestingly, reduction in *DmelPex1* transcripts by RNAi in S2 cells produced a cytosolic signal and eliminated the green punctate pattern of GFP-SKL. It is possible that *DmelPex1* has a similar function in peroxisome biogenesis and assembly as in other organisms.

RNAi was performed against two different regions of the *DmelPex6* gene. RNAi against *DmelPex6(i)* produced an increased number of small green punctate structures. On the other hand, RNAi against *DmelPex6(ii)* produced both a cytosolic signal for GFP-SKL and an increased number of small green punctate structures. This is very interesting as this gene has alternatively spliced isoforms. It is possible that dsRNA selectively targeted different regions of the mRNA that were encoded by alternative splice isoforms. These isoforms could have different stability, hence dsRNAi-mediated knock-down of this gene produced two types of phenotypes.

Pex2p, Pex12p, Pex13p, and Pex14p have been shown to be required for docking and translocation of receptor-cargo complexes (Purdue and Lazarow, 2001). Individual dsRNAi-mediated knock-down of these genes produced a reduction in the number of punctate structures containing GFP-SKL and/or a strong cytosolic signal for GFP-SKL. Several studies have shown that Pex2p and Pex12p are involved in translocation and are required for peroxisomal matrix protein import (Chang et al., 1997; 1999; Eckert and Johnsson, 2003). Reduction in *DmelPex2* and *DmelPex12* transcripts by RNAi produced a cytosolic signal for GFP-SKL. Interestingly, our results are consistent with the data obtained from the mutants for *PEX2* and *PEX12* in other organisms (Chang et al., 1997; Albertini et al., 2001).

Pex13p and Pex14p are conserved from yeasts to human stressing their importance in peroxisome biogenesis (reviewed in Heiland and Erdmann, 2005). Pex13p, Pex14p and Pex17p are the docking proteins for the PTS1 and PTS2 receptors at the peroxisomal membrane. It is interesting to note that RNAi against the *DmelPex13* and *DmelPex14* genes produced a strong cytosolic signal for GFP-SKL.

Several studies have shown that Pex19p is required for peroxisomal membrane formation, and it has been suggested to function as both receptor and chaperone that binds and stabilizes newly synthesised peroxisomal membrane proteins at the peroxisome membrane (Pinto et al., 2006; Fujiki et al., 2006). It has also been implied that Pex19p interacts with Pex3p in the endoplasmic reticulum (ER) during the early stages of de *novo* peroxisome biogenesis and is

required for the exit of Pex3p from the ER (Hoepfner et al., 2005; Tam et al., 2005). The absence of Pex19p in all organisms studied so far leads to their inability to assemble peroxisomes and to the lack of identifiable peroxisomal membrane structures (Hettema et al., 2000). Very interestingly, dsRNAi-mediated knock-down of the putative *D. melanogaster* homolog of *PEX19* (*DmelPex19*) did not prevent the assembly of peroxisomes in S2 cells. However, it produced a phenotype with 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 against *DmelPex19* could arise due to a variety of reasons. First, that the identified *DmelPex19* is not a true *D. melanogaster* homolog of *PEX19* gene. Second, the DmelPex19 protein may have a different function in peroxisome biogenesis as compared to the Pex19 protein in other organisms. Third, *D. melanogaster* may have another protein whose function in peroxisome biogenesis and peroxisomal membrane formation is redundant to that of DmelPex19 protein. Fourth, RNAi reduction of *DmelPex19* transcript may have been insufficient to give a loss-of-function phenotype.

PEX11 is conserved among eukaryotes and controls peroxisome division and proliferation (Li et al., 2002). Cells with deletion and/or mutation of *PEX11* genes have decreased numbers of enlarged peroxisomes (Erdmann and Blobel, 1995). S2 cells treated with dsRNAi against the *DmelPex11* gene had few and enlarged peroxisomes but were unaffected in their ability to import GFP-SKL into

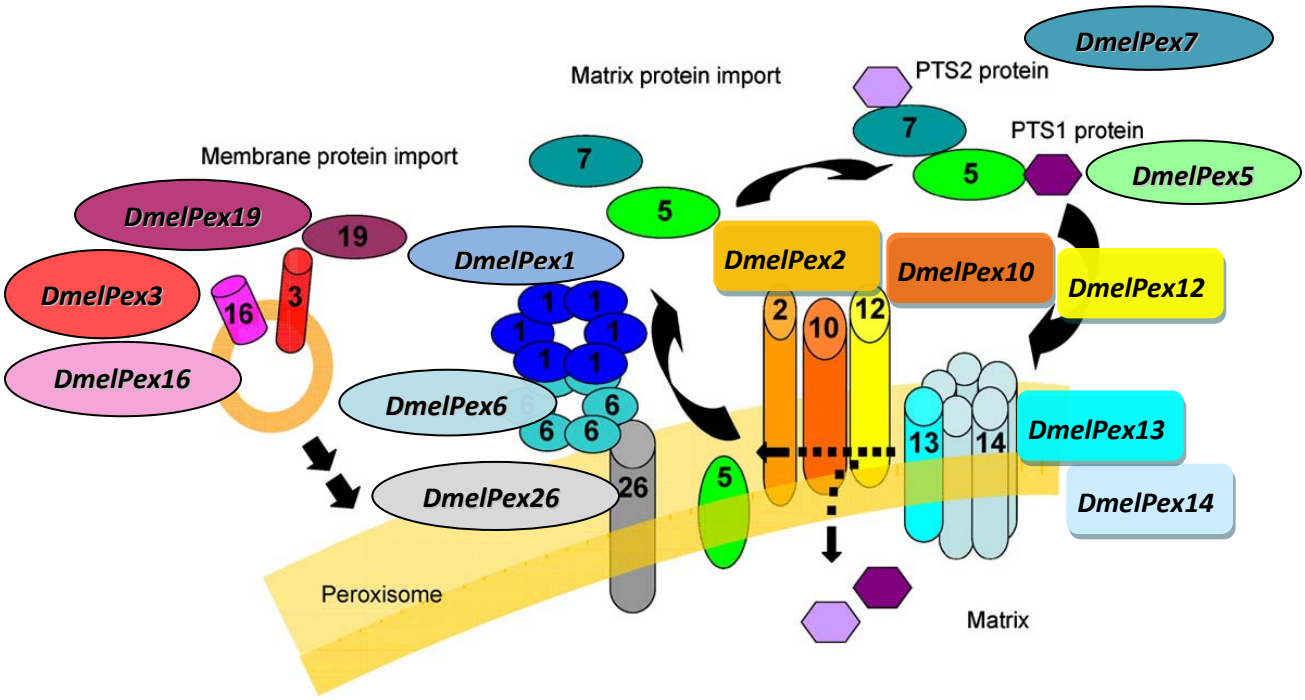

Figure 13. Model for peroxisomes biogenesis. The early peroxins PEX3, PEX16 and PEX19 are required during the initial stages of peroxisomal membrane formation and/or assembly. PEX5 and PEX7 are the shuttling receptors that recognise cytosolic peroxisomal matrix proteins containing a PTS1 or PTS2, respectively. The docking and translocation of receptor-cargo complexes at the peroxisomal membrane are facilitated by a docking complex (PEX13, PEX14 and PEX17) and a translocation complex (PEX2, PEX10 and PEX12). Peroxins implicated in the recycling of matrix protein receptors (PTS1 and PTS2) to the cytosol are PEX1, PEX6, and PEX26 (as modified form Steinberg et al., 2006).

peroxisomes. This suggests that *DmelPex11* probably functions similarly to *PEX11* genes in other organisms in controlling peroxisome proliferation and division.

DmelPex20p and DmelPex31p have little similarity to *Y. lipolytica* Pex20p and *S. cerevisiae* Pex31p. Nevertheless, DmelPex20p and DmelPex31p appear to function in peroxisome biogenesis like their yeast homologs. This demonstrates that despite the evolutionary distance between flies and yeast, *D. melanogaster* has apparently retained *PEX* genes heretofore only reported in yeasts and maintained their activity in peroxisome biogenesis.

RNAi against the putative *D. melanogaster* homolog of fungal *PEX20* did not produce a cytosolic signal for GFP-SKL and had no affect on the targeting of GFP-SKL to peroxisomes in S2 cells. This was an expected phenotype because it has been shown that Pex20 protein has a chaperoning function in the targeting of PTS2-containing, but not PTS1-containing, proteins to peroxisomes (Titorenko et al., 1998). Hence, reduction of *DmelPex20* transcripts by RNAi did not affect the targeting of GFP-SKL, which contains the PTS1, to peroxisomes.

The Pex30 family of proteins includes Pex30p, Pex31p and Pex32p and functions in controlling peroxisome number and size in different yeast species. S2 cells treated with RNAi against the *DmelPex31* gene had an increased number of small peroxisomes as compared to control S2 cells and no mislocalization of GFP-SKL to the cytosol. Similarly, absence of Pex31p in *S. cerevisiae* cells did not mislocalize PTS1-containing proteins to the cytosol; however, in contrast to the situation in S2 cells, gave rise to reduced numbers of enlarged peroxisomes

when compared to wild-type cells (Vizeacoumar et al., 2004). Therefore, even though the DmelPex31 protein may be similar to *S. cerevisiae* Pex31p, it may function more like *S. cerevisiae* Pex30p which, when absent, results in increased numbers of small peroxisomes (Vizeacoumar et al., 2004). Nevertheless, *DmelPex31* most likely functions in the control of peroxisome size and number.

Interestingly, RNAi against many *PEX* genes that are essential for peroxisome biogenesis (like *PEX1*, *PEX3*, or *PEX16*) did not eliminate all peroxisomes in S2 cells. This could be due to a variety of reasons. Firstly, it is important to note that RNAi is a method of gene knock-down and not knock-out. Secondly, in some S2 cells, the small amount of residual protein that remained after RNAi treatments may have been sufficient to fulfill cellular roles. Thirdly, reduction of some transcripts by RNAi may have been insufficient to give a loss-of-function phenotype due to long protein life and/or high endogenous expression. Fourthly, in the absence of some *PEX* genes (those mainly involved in biogenesis), the already formed or pre-existing peroxisomes may have been functional and possibly, once formed, divided and proliferated on their own for many generations.

Overall, RNAi analysis in S2 cells demonstrates that *D. melanogaster* contains a number of homologs of *PEX* genes whose encoded peroxins perform functions in peroxisome biogenesis similar to those of their peroxin homologs in other organisms (Fig. 13). Also, a recent paper published early this year reported the identification of some *Drosophila PEX* genes that were examined in this study (Chen et al., 2010). This further shows that peroxisome biogenesis in *D.*

melanogaster is mechanistically similar to peroxisome biogenesis in other organisms including humans, thus providing evidence that *D. melanogaster* can serve as a valid model system with which to investigate the effects of compromised peroxisome assembly on development of a multicellular organism.

4.2. *D. melanogaster* as a model system for the human peroxisome biogenesis disorders

Peroxisomes are essential for normal human development and physiology. Loss of global peroxisomal function because of the inability to assemble peroxisomes is associated with a number of diseases ranging from relatively mild single enzyme deficiencies to severe PBDs. PBDs are lethal genetic conditions that are not compatible with life or normal development (Fujiki, 2000). Mutations in 13 *PEX* genes are the basis of the human PBDs, including ZS (Steinberg et al., 2006; Shimozawa, 2007).

The *PEX1* gene encodes a member of the AAA-family of ATPases and is essential for peroxisome biogenesis (Portsteffen et al., 1997; Reuber et al., 1997). It is the causative gene for peroxisomal disorders of complementation group 1 (CG1), and expression of human *PEX1* is able to rescue the peroxisomal biogenesis defect of human fibroblasts isolated from patients in CG1 (Portsteffen et al., 1997; Reuber et al., 1997). Mutations in the *PEX1* gene are the most common cause of ZS (Reuber et al., 1997; Steinberg et al., 2006). Patients with ZS suffer from severe symptoms including mental retardation, neuronal defects, hepatic dysfunction, seizures, liver cysts, poor feeding, renal abnormalities, hypotonia, as well as musculoskeletal defects (Crane et al., 2005; Rosewich et al.,

2005; Fujiki et al., 2008). These patients are significantly impaired, exhibit developmental delay, and die early in infancy (reviewed in Shimozawa, 2007). We decided to study the function of *DmelPex1* in *D. melanogaster* development because of the prominence of *PEX1* in the PBDs, the severity of ZS, and the results of S2 cells studies that showed *DmelPex1* has an important role in peroxisome formation.

Developmental assays showed that *DmelPex1* mutant flies failed to develop past the larval stage. Mutant larvae were smaller in size, failed to move, crawl, and/or feed effectively as compared to their wild-type counterparts. Also, mutant larvae 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. These phenotypes of *DmelPex1* mutant flies were very similar to that of ZS infants who have reduced growth, poor feeding, profound neuronal abnormalities and early death (Steinberg et al., 2006).

Interestingly, the ZS patients and *DmelPex1* mutant flies shared more pathological phenotypes. In *D. melanogaster*, glial cells provide support and insulation to the axons of the CNS and PNS similar to myelination by oligodendrocytes and Schwann cells in vertebrates (Banerjee et al., 2006a,b; Banerjee and Bhat, 2007; Freeman and Doherty, 2006). *DmelPex1* mutant flies had extensively disorganized glial cells. This is similar to ZS patients who have severely demyelinated axons in both the CNS and PNS (Powers et al., 1985; Steinberg et al., 2006). Muscle development in *DmelPex1* mutant embryos was not affected, and both mutants and wild-type embryos exhibited normal

musculature. Therefore, the lack of effective movement and feeding behaviors in *DmelPex1* mutant animals was most likely due to neuronal defects.

In humans, peroxisomes are mainly abundant in the liver and kidney for detoxification. ZS patients show structural abnormalities in the kidney (Steinberg et al., 2006). In insects, the malpighian tubule performs similar functions as the mammalian kidney. It is a system of blind-ending tubules that is involved in excretion of uric acid. The malpighian tubules of *DmelPex1* mutants were severely deformed, indicating a renal abnormality similar to humans suffering from ZS. All these results show that *DmelPex1* is essential for the normal development of *D. melanogaster*, and deleting this gene in flies produces several pathological phenotypes that are similar to those exhibited by ZS patients. Overall, this study indicates that *D. melanogaster* can be used as a model system for the human PBDs.

D. melanogaster is one of the most popular model organisms and is used extensively in genetic and developmental studies. In mammals and fuitflies, the guiding principles of axon pathfinding and neurogenesis are very similar (Wu et al., 2008). In recent years, flies have become an invaluable model system for the study of neurodegenerative disorders because they provide a platform for genetic screens to identify components of pathological pathways. Use of *D. melanogaster* as a metazoan model for the PBDs could be useful for the overall understanding of molecular and biochemical pathways that cause demyelination in ZS patients and for defining novel treatments to prevent and/or cure these diseases.

4.3. Relevance of this study

The aim of this study was to find peroxisome-related genes in the fruitfly *D. melanogaster* and to use it as a model system for peroxisome research, particularly for the human PBDs. Characterization of *DmelPex* genes using dsRNAi analysis showed that peroxisome assembly in *D. melanogaster* is mechanistically similar to peroxisome assembly in other eukaryotes. Furthermore, the developmental and neuronal abnormalities arising in *D. melanogaster* from mutation of the *DmelPex1* gene mimicked the global developmental defects observed in ZS patients. On the whole, this study makes a compelling argument for the use of *D. melanogaster* as a valid and tractable model with which to investigate the PBDs and for the use of *D. melanogaster* peroxisome assembly mutants to screen for possible therapeutics for intervention in the PBDs.

This is significant because *D. melanogaster* as a model system for the human PBDs can help us to better understand the biochemical mechanisms of these diseases by employing systems like the GAL4-UAS system and GAL4-targeted FLP/FRT recombination (Brand and Dormand, 1995; Theodosiou and Xu, 1998). With the help of these techniques this disease model can be genetically treated by expressing specific *DmelPex* genes at a specific time in a specific tissue. In the future, expressing *DmelPex1* in various tissues of the mutant animal can help to identify specific tissues that require peroxisomes for normal development in flies. Moreover, it will help to identify pathways involved in the progression of the PBDs and to hunt for suppressor mutations that might prevent or delay PBD

symptoms. Subsequent research could have a future application for designing therapeutics in the treatment of PBDs.

This study is a stepping stone for the use of *D. melanogaster* as a powerful tool for understanding the molecular and physiological defects underlying the human peroxisomal disorders. This is novel and very exciting as it opens a wide field for PBD-related research in the future. For example, to understand if a change in diet delays the disease and/or rescues the development of *DmelPex1* mutant flies, they can be treated using chemical methods by providing different carbon and lipid sources. A recent study showed that very-long chain fatty acids accumulate in several *DmelPex* mutant flies (Chen et al., 2010). Also, tools like the misexpression system can be used to misexpress various human *PEX* genes in the fly at particular times and tissues to understand the similarity between human and fly *PEX* genes and if one can compensate for the other.

Investigation of more *DmelPex* gene mutant flies from the previous work in S2 cells will help to understand the role of various *PEX* genes in peroxisome biogenesis in the whole multicellular organism during development. These subsequent studies could have an enormous application for the treatment of PBDs. In the future, with the help of powerful genetic, cellular, and molecular tools, *D. melanogaster* could provide a model system for drug discovery in PBD research. The hope is that by studying PBDs in flies, we could accelerate genetic research in neuronal and other ZS-related defects in PBD patients.[6]

[6] This thesis is based on a paper submitted for publication.

CHAPTER 5: REFERENCES

Albertini, M., W. Girzalsky, M. Veenhuis, and W. H. Kunau. 2001. Pex12p of *Saccharomyces cerevisiae* is a component of a multi-protein complex essential for peroxisomal matrix protein import. *Eur. J. Cell Biol*. 80:257-270.

Aubourg, P. 2007. Axons need glial peroxisomes. *Nat. Genet.* 8:936-938.

Banerjee, S., and M. A. Bhat. 2007. Neuron-glial interactions in blood–brain barrier formation. *Annu. Rev. Neurosci*. 30:235-258.

Banerjee, S., and M. A. Bhat. 2008. Glial ensheathment of peripheral axons in *Drosophila. J. Neurosci. Res*. 86:1189-1198.

Banerjee, S., A. M. Pillai, R. Paik, J. Li, and M. A. Bhat. 2006. Axonal ensheathment and septate junction formation in the peripheral nervous system of *Drosophila. J. Neurosci*. 26:3319-3329.

Beard, M. E., and E. Holtzman. 1987. Peroxisomes in wild-type and rosy mutant *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 84:7433-7437.

Bilen, J., and N. M. Bonini. 2005. *Drosophila* as a model for human neurodegenerative disease. *Annu. Rev. Genet*. 39:153-171.

Brand, A. H., and E. L. Dormand. 1995. The GAL4 system as a tool for unraveling the mysteries of the *Drosophila* nervous system. *Curr. Opinion Neurobiol.* 5:572-578.

Brown, L. A., and A. Baker. 2003. Peroxisome biogenesis and the role of protein import. *J. Cell Mol. Med*. 7:388-400.

Brown, T. W., V. I. Titorenko, and R. A. Rachubinski. 2000. Mutants of the *Yarrowia lipolytica PEX23* gene encoding an integral peroxisomal membrane peroxin mislocalize matrix proteins and accumulate vesicles

containing peroxisomal matrix and membrane proteins. *Mol. Biol. Cell* 11:141-152.

Cauchi, R. J., and M. van den Heuvel. 2006. The fly as a model for neurodegenerative diseases: is it worth the jump? *Neurodegener. Dis*. 3:338-356.

Chang, C. C., W. H. Lee, H. Moser, D. Valle, and S. J. Gould. 1997. Isolation of the human PEX12 gene, mutated in group 3 of the peroxisome biogenesis disorders. *Nat. Genet.* 15:385-388.

Chang, C. C., D. S. Warren, K. A. Sacksteder, and S. J. Gould. 1999. PEX12 interacts with PEX5 and PEX10 and acts downstream of receptor docking in peroxisomal matrix protein import. *J. Cell Biol.* 147:761-774.

Chen, H., Z. Liu, and X. Huang. 2010. Drosophila models of peroxisomal biogenesis disorder: peroxins are required for spermatogenesis and very-long-chain fatty acid metabolism. *Hum. Mol. Genet.* 19:494-505.

Clemens, J. C., C. A. Worby, N. Simonson-Leff, M. Muda, T. Maehama, B. A. Hemmings, and J. E. Dixon. 2000. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. USA* 97:6499-6503.

Cooper, G. M. 2000. The Cell – A Molecular Approach. *Sinauer Associates, Sunderland.* Second edition. 690 pp.

Crane, D. I., M. A. Maxwell, and B. C. Paton. 2005. Pex1 mutations in the Zellweger spectrum of the peroxisome biogenesis disorders. *Hum. Mutat.* 26:167-175.

de Duve, C., and P. Baudhuin. 1966. Peroxisomes (microbodies and related particles). *Physiol. Rev*. 46:323-357.

de Graaf, I. M., E. Pajkrt, M. Keessen, N. J. Leschot, and C. M. Bilardo. 1999. Enlarged nuchal translucency and low serum protein concentration as

possible markers for Zellweger syndrome. *Ultrasound Obstet. Gynecol*. 13:268-270.

Dixit, E., S. Boulant, Y. Zhang, A. S. Lee, C. Odendall, B. Shum, N. Hacohen, Z. J. Chen, S. P. Whelan, M. Fransen, M. L. Nibert, G. Supert-Furga, and J. C. Kagan. 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* 141:668-681.

Echeverri, C. J., and N. Perrimon. 2006. High-throughput RNAi screening in cultured cells: a user's guide. *Nat. Rev. Genet.* 7:373-384.

Eckert, J. H., and N. Johnsson. 2003. Pex10p links the ubiquitin conjugating enzyme Pex4p to the protein import machinery of the peroxisome. *J. Cell Sci.* 116:3623-3634.

Eitzen, G. A., R. K. Szilard, and R. A. Rachubinski. 1997. Enlarged peroxisomes are present in oleic acid-grown *Yarrowia lipolytica* overexpressing the *PEX16* gene encoding an intraperoxisomal peripheral membrane peroxin. *J. Cell Biol*. 137:1265-1278.

Erdmann, R., and G. Blobel. 1995. Giant peroxisomes in oleic acid-induced *Saccharomyces cerevisiae* lacking the peroxisomal membrane protein Pmp27. *J. Cell Biol*. 128:509-523.

Erdmann, R., F. F. Wiebel, A. Flessau, J. Rytka, A. Beyer, K. U. Fröhlich, and W. H. Kunau. 1991. *PAS1*, a yeast gene required for peroxisome biogenesis, encodes a member of a novel family of putative ATPases. *Cell* 64:499-510.

Erdmann, R., M. Veenhuis, D. Mertens, and W. H. Kunau. 1989. Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 86:5419-5423.

Fagarasanu, A., M. Fagarasanu, and R. A. Rachubinski. 2007. Maintaining peroxisome populations: a story of division and inheritance. *Annu. Rev. Cell Dev. Biol.* 23:321-344.

Foley, E., and P. H. O'Farrell. 2004. Functional dissection of an innate immune response by a genome-wide RNAi screen. *PLoS Biol*. 2:E203.

Freeman, M. R., and J. Doherty. 2006. Glial cell biology in *Drosophila* and vertebrates. *Trends Neurosci*. 29:82-90

Fujiki, Y. 2000. Peroxisome biogenesis and peroxisome biogenesis disorders. *FEBS Lett.* 476:42-46.

Fujiki, Y., N. Miyata, N. Matsumoto, and S. Tamura. 2008. Dynamic and functional assembly of the AAA peroxins, Pex1p and Pex6p, and their membrane receptor Pex26p involved in shuttling of the PTS1 receptor Pex5p in peroxisome biogenesis. *Biochem. Soc. Trans*. 36:109-113.

Fujiki, Y., Y. Matsuzono, T. Matsuzaki, and M. Fransen. 2006. Import of peroxisomal membrane proteins: the interplay of Pex3p- and Pex19p-mediated interactions. *Biochim. Biophys. Acta* 1763:1639-1646.

Furuki, S., S. Tamura, N. Matsumoto, N. Miyata, A. Moser, H. W. Moser, and Y. Fujiki. 2006. Mutations in the peroxin Pex26p responsible for peroxisome biogenesis disorders of complementation group 8 impair its stability, peroxisomal localization, and interaction with the Pex1p-Pex6p complex. *J. Biol. Chem.* 281:1317-1323.

Gould, S. J., and D. Valle. 2000. Peroxisome biogenesis disorders: genetics and cell biology. *Trends Genet*. 16:340-345.

Gould, S. J., G. V. Raymond, and D. Valle. 2001. The peroxisome biogenesis disorders. IN: The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) *McGraw Hill, New York*. 8th Ed. pp. 3181-3217.

Hawkins, J., D. Mahony, S. Maetschke, M. Wakabayashi, R. D. Teasdale, and M. Bodén. 2007. Identifying novel peroxisomal proteins. *Proteins* 69:606-616.

Heiland, I., and R. Erdmann. 2005. Biogenesis of peroxisomes. Topogenesis of the peroxisomal membrane and matrix proteins. *FEBS J*. 272:2362-2372.

Hettema, E. H., W. Girzalsky, M. van den Berg, R. Erdmann, and B. Distel. 2000. *Saccharomyces cerevisiae* Pex3p and Pex19p are required for proper localization and stability of peroxisomal membrane proteins. *EMBO J*. 19:223-233.

Hoepfner, D., D. Schildknegt, I. Braakman, P. Philippsen, and H. F. Tabak. 2005. Contribution of the endoplasmic reticulum to peroxisome formation. *Cell* 122:85-95.

Hughes, S. C., and H. M. Krause. 1999. Single and double FISH protocols for *Drosophila*. *Methods Mol. Biol*. 122:93-101.

Kiel, J. A. W., M. Veenhuis, and I. J. van der Klei. 2006. *PEX* genes in fungal genomes: common, rare or redundant. *Traffic* 7:1291-1303.

Kim, H., S. C. Ling, G. C. Rogers, C. Kural, P. R. Selvin, S. L. Rogers, and V. I. Gelfand. 2007. Microtubule binding by dynactin is required for microtubule organization but not cargo transport. *J. Cell Biol*. 176:641-651.

Kural, C., H. Kim, S. Syed, G. Goshima, V. I. Gelfand, and P. R. Selvin. 2005. Kinesin and dynein move a peroxisome in vivo: a tug-of-war or coordinated movement? *Science*. 308:1469-1472.

Lambkin, G. R., and R. A. Rachubinski. 2001. *Yarrowia lipolytica* cells mutant for the peroxisomal peroxin Pex19p contain structures resembling wild-type peroxisomes. *Mol. Biol. Cell* 12:3353-3364.

Lazarow, P. B. 2006. The import receptor Pex7p and the PTS2 targeting sequence. *Biochim. Biophys. Acta* 1763:1599-1604.

Lazarow, P. B., and Y. Fujiki. 1985. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol*. 1:489-530.

Li, X., and S. J. Gould. 2002. PEX11 promotes peroxisome division independently of peroxisome metabolism. *J. Cell Biol*. 156:643-651.

Li, X., E. Baumgart, G. X. Dong, J. C. Morrell, G. Jimenez-Sanchez, D. Valle, K. D. Smith, and S. J. Gould. 2002. PEX11α is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor alpha-mediated peroxisome proliferation. *Mol. Cell. Biol*. 22:8226-8240.

Marzioch, M., R. Erdmann, M. Veenhuis, and W. H. Kunau. 1994. *PAS7* encodes a novel yeast member of the WD-40 protein family essential for import of 3-oxoacyl-CoA thiolase, a PTS2-containing protein, into peroxisomes. *EMBO J.* 13:4908-4918.

Maxwell, M. A., P. V. Nelson, S. J. Chin, B. C. Paton, W. F. Carey, and D. I. Crane. 1999. A common PEX1 frameshift mutation in patients with disorders of peroxisome biogenesis correlates with the severe Zellweger syndrome phenotype. *Hum. Genet.* 105:38-44.

Maxwell, M. A., T. Allen, P. Solley, T. Svingen, B. C. Paton, and D. I. Crane. 2002. Novel PEX1 mutations and genotype-phenotype correlations in Australasian peroxisome biogenesis disorder patients. *Hum. Mut*. 20:342-351.

Petriv, O. I., D. B. Pilgrim, R. A. Rachubinski, and V. I. Titorenko. 2002. RNA interference of peroxisome-related genes in *C. elegans*: a new model for human peroxisomal disorders. *Physiol. Genomics.* 10:79-91.

Pinto, M. P., C. P. Grou, I. S. Alencastre, M. E. Oliveira, C. Sa-Miranda, M. Fransen, and J. E. Azevedo. 2006. The import competence of a peroxisomal membrane protein is determined by Pex19p before the docking step. *J. Biol. Chem*. 281:34492-34502.

Platta, H. W., and R. Erdmann. 2007. Peroxisomal dynamics. *Trends Cell Biol*. 17:474-484.

Platta, H. W., S. Grunau, K. Rosenkranz, W. Girzalsky, and R. Erdmann. 2005. Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol. *Nat. Cell Biol*. 8:817-822.

Poll-Thé, B. T., J. Gootjes, M. Duran, J. B. C. de Klerk, L. J. Wenniger-Prick, R. J. C. Admiraal, H. R. Waterham, R. J. A. Wanders, and P. G. Barth. 2004. Peroxisome biogenesis disorders with prolonged survival: phenotypic expression in a cohort of 31 patients. *Am. J. Med. Genet.* 126A:333-338.

Portsteffen, H., A. Beyer, E. Becker, C. Epplen, A. Pawlak, W. H. Kunau, and G. Dodt. 1997. Human PEX1 is mutated in complementation group 1 of the peroxisome biogenesis disorders. *Nat. Genet.* 17:449-452.

Powers, J. M., and H. W. Moser. 1998. Peroxisomal disorders: genotype, phenotype, major neuropathologic lesions, and pathogenesis. *Brain Pathol*. 8:101-120.

Powers, J. M., H. W. Moser, A. B. Moser, J. K. Upshur, B. F. Bradford, S. G. Pai, P. H. Kohn, J. Frias, and C. Tiffany. 1985. Fetal cerebrohepatorenal (Zellweger) syndrome: dysmorphic, radiologic, biochemical, and pathologic findings in four affected fetuses. *Hum. Pathol*. 16:610-620.

Purdue, P. E., and P. B. Lazarow. 2001. Peroxisome biogenesis. *Annu. Rev. Cell Dev. Biol.* 17:701-752.

Purdue, P. E., M. Skoneczny, X. Yang, J. W. Zhang, and P. B. Lazarow. 1999. Rhizomelic chondrodysplasia punctata, a peroxisomal biogenesis disorder caused by defects in Pex7p, a peroxisomal protein import receptor: a minireview. *Neurochem. Res*. 24:581-586.

Rachubinski, R. A., and S. Subramani. 1995. How proteins penetrate peroxisomes. *Cell* 83:525-528.

Raymond, F. L., and P. Tarpey. 2006. The genetics of mental retardation. *Hum. Mol. Genet*. 15:R110-R116.

Reiter, L. T., L. Potocki, S. Chien, M. Gribskov, and E. Bier. 2001. A systematic analysis of human disease-associated gene sequences in *Drosophila melanogaster*. *Genome Res*. 11:1114-1125.

Reuber, B. E., E. Germain-Lee, C. S. Collins, J. C. Morrell, R. Ameritunga, H. W. Moser, D. Valle, and S. J. Gould. 1997. Mutations in *PEX1* are the most common cause of peroxisome biogenesis disorders. *Nat. Genet*. 17:445-448.

Rosewich, H., A. Ohlenbusch, and J. Gartner. 2005. Genetic and clinical aspects of Zellweger spectrum patients with PEX1 mutations. *J. Med. Genet.* 42:e58.

Sacksteder, K. A., and S. J. Gould. 2000. The genetics of peroxisome biogenesis. *Annu. Rev. Genet*. 34:623-652.

Sanchez-Martinez, A., N. Luo, P. Clemente, C. Adan, R. Hernandez-Sierra, P. Ochoa, M. A. Fernandez-Moreno, L. S. Kaguni, and R. Garesse. 2006. Modeling human mitochondrial diseases in flies. *Biochim. Biophys. Acta* 1757:1190-1198.

Schell-Steven, A., K. Stein, M. Amoros, C. Landgraf, R. Volkmer-Engert, H. Rottensteiner, and R. Erdmann. 2005. Identification of a novel, intraperoxisomal Pex14-binding site in Pex13: association of Pex13 with the docking complex is essential for peroxisomal matrix protein import. *Mol. Cell. Biol*. 25:3007-3018.

Schliebs, W., and W. H. Kunau. 2004. Peroxisome membrane biogenesis: the stage is set. *Curr. Biol*. 14:R397-R399.

Schluter, A., S. Fourcade, R. Ripp, J. L. Mandel, O. Poch, and A. Pujol. 2006. The evolutionary origin of peroxisomes: an ER-peroxisome connection. *Mol. Biol. Evol*. 23:838-845.

Schrader, M., and H. D. Fahimi. 2008. The peroxisome: still a mysterious organelle. *Histochem. Cell Biol*. 129:421-440.

Shimozawa. N. 2007. Molecular and clinical aspects of peroxisomal diseases. *J. Inherit. Metab. Dis*. 30:193-197.

Shimozawa, N., T. Nagase, Y. Takemoto, M. Funato, N. Kondo, and Y. Suzuki. 2005. Molecular and neurologic findings of peroxisome biogenesis disorders. *J. Child Neurol*. 20:326-329.

Singh, I. 1997. Biochemistry of peroxisomes in health and disease. *Mol. Cell Biochem*. 167:1-29.

Southall, T. D., S. Terhzaz, P. Cabrero, V. R. Chintapalli, J. M. Evans, J. A. T. Dow and S. A. Davies. 2006. Novel subcellular locations and functions for secretory pathway Ca^{2+}/Mn^{2+}-ATPases. *Physiol. Genom*. 26:35-45.

Steinberg, S. J., G. Dodt, G. V. Raymond, N. E. Braverman, A. B. Moser, and H. W. Moser. 2006. Peroxisome biogenesis disorders. *Biochim. Biophys. Acta* 1763:1733-1748.

St. Jules, R., M. Beard, and E. Holtzman. 1989. Cytochemical localization of a D-amino acid oxidizing enzyme in peroxisomes of *Drosophila melanogaster*. *Tissue Cell* 21:661-671.

St. Jules, R., W. Setlik, J. Kennard, and E. Holtzman. 1990. Peroxisomes in the head of *Drosophila melanogaster*. *Exp. Eye Res.* 51:607-617.

Subramani, S. 1993. Protein import into peroxisomes and biogenesis of the organelle. *Annu. Rev. Cell Biol.* 9:445-478.

Subramani, S. 1997. *PEX* genes on the rise. *Nat. Genet*. 15:331-333.

Subramani, S., A. Koller, and W. B. Snyder. 2000. Import of peroxisomal matrix and membrane proteins. *Annu. Rev. Biochem*. 69:399-418.

Tamura, S., N. Shimozawa, Y. Suzuki, T. Tsukamoto, T. Osumi, and Y. Fujiki. 1998. A cytoplasmic AAA family peroxin, Pex1p, interacts with Pex6p. *Biochem. Biophys. Res. Commun*. 3:883-886.

Tam, Y. Y. C., A. Fagarasanu, M. Fagarasanu, and R. A. Rachubinski. 2005. Pex3p initiates the formation of a preperoxisomal compartment from a subdomain of the endoplasmic reticulum in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280:34933-34939.

Terlecky, S. R., W. M. Nuttley, D. McCollum, E. Sock, and S. Subramani. 1995. The *Pichia patoris* peroxisomal protein PAS8p is the receptor for the C-terminal tripeptide peroxisomal targeting signal. *EMBO J.* 14:3627-3634.

Theodosiou, N. A., and T. Xu. 1998. Use of FLP/FRT system to study *Drosophila* development. *Methods* 14:355-365.

Titorenko, V. I., J. J. Smith, R. K. Szilard, and R. A. Rachubinski. 1998. Pex20p of the yeast *Yarrowia lipolytica* is required for the oligomerization of thiolase in the cytosol and for its targeting to the peroxisome. *J. Cell Biol.* 142:403-420.

Titorenko, V. I., and R. A. Rachubinski. 2001a. Dynamics of peroxisome assembly and function. *Trends Cell Biol*. 11:22-29.

Titorenko, V. I., and R. A. Rachubinski. 2001b. The life cycle of the peroxisome. *Nat. Rev. Mol. Cell Biol.* 2:357-368.

Tolbert, N. E., and E. Essner. 1981. Microbodies: peroxisomes and glyoxysomes. *J. Cell Biol.* 91:271s-283s.

van den Bosch, H., R. B. H. Schutgens, R. J. A. Wanders, and J. M. Tager. 1992. Biochemistry of peroxisomes. *Annu. Rev. Biochem*. 61:157-197.

van der Leij, I., M. van den Berg, R. Boot, M. Franse, B. Distel, and H. F. Tabak. 1992. Isolation of peroxisome assembly mutants from *Saccharomyces cerevisiae* with different morphologies using a novel positive selection procedure. *J. Cell Biol*. 119:153-162.

Vizeacoumar, F. J., J. C. Torres-Guzman, D. Bouard, J. D. Aitchison, and R. A. Rachubinski. 2004. Pex30p, Pex31p, and Pex32p form a family of

peroxisomal integral membrane proteins regulating peroxisome size and number in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 15:665-677.

Walter, C., J. Gootjes, P. A.Mooijer, H. Portsteffen, C. Klein, H. R. Waterham, P. G. Barth, J. T. Epplen, W. H. Kunau, R. J. A. Wanders, and G. Dodt. 2001. Disorders of peroxisome biogenesis due to mutations in PEX1: phenotypes and PEX1 protein levels. *Am. J. Hum. Genet*. 69:35-48.

Wanders, R. J. A. 1999. Peroxisomal disorders: clinical, biochemical, and molecular aspects. *Neurochem. Res*. 24:565-580.

Wanders, R. J. A., and J. M. Tager. 1998. Lipid metabolism in peroxisomes in relation to human disease. *Mol. Aspects Med*. 19:69-154.

Wanders, R. J. A., and H. R. Waterham. 2006. Biochemistry of mammalian peroxisomes revisited. *Annu. Rev. Biochem*. 75:295-332.

Wanders, R. J. A, P. G. Barth, R. B. H. Schutgens, and H. S. A. Heymans. 1996. Peroxisomal disorders: post- and prenatal diagnosis based on a new classification with flowcharts. *Int. Pediatr*. 11:203-214.

Wanders, R. J. A., P. Vreken, S. Ferdinandusse, G. A. Jansen, H. R. Waterham, C. W. van Roermund, and E. G. van Grunsven. 2001. Peroxisomal fatty acid α- and β-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. *Biochem. Soc. Trans.* 29:250-267.

Weller, S., S. J. Gould, and D. Valle. 2003. Peroxisome biogenesis disorders. *Annu. Rev. Genomics Hum. Genet*. 4:165-211.

Wu, P. S., B. Egger, and A. H. Brand. 2008. Asymmetric stem cell division: lessons from *Drosophila. Semin. Cell Dev. Biol*. 19:283-293.

Yagi, S., and H. Ogawa. 1996. Effect of tryptophan metabolites on fluorescent granules in the Malpighian tubules of eye color mutants of *Drosophila melanogaster*. *Zoolog. Sci.* 13:97-104.