Modeling human peroxisome biogenesis disorders in the nematode *Caenorhabditis elegans*

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Summary

Peroxisomes are ubiquitous eukaryotic organelles. The proteins required for peroxisome biogenesis are called peroxins, and mutations in the peroxin genes cause the devastating human developmental syndromes called the peroxisome biogenesis disorders. Our interest is in elaborating the roles that peroxisomes play in *Caenorhabditis elegans* development, and in establishing an invertebrate model system for the human peroxisome biogenesis disorders. The genome of *C. elegans* encodes homologs of 11 of the 13 human peroxins. We disrupted five nematode peroxins using RNA interference (RNAi) and found that RNAi knockdown of each one causes an early larval arrest at the L1 stage. Using a green fluorescent protein reporter targeted to the peroxisome, we establish that peroxisomal import is impaired in *prx-5(RNAi)* nematodes. *prx-5(RNAi)* animals are blocked very early in the L1 stage and do not initiate normal postembryonic cell divisions, similar to starvation-arrested larvae. Cell and axonal migrations that normally occur during the L1 stage also appear blocked. We conclude that peroxisome function is required for *C. elegans* postembryonic development and that disruption of peroxisome assembly by *prx-5(RNAi)* prevents scheduled postembryonic cell divisions. Defects in the cellular localization of peroxisomal proteins and in development are shared features of human and nematode peroxisome biogenesis disorders. In setting up a *C. elegans* model of peroxisomal biogenesis disorders, we suggest that genetic screens for suppression of the Prx developmental block will facilitate identification of novel intervention strategies and may provide new insights into human disease pathogenesis.

Key words: Zellweger syndrome model, Peroxin, Peroxisome biogenesis

Introduction

Virtually all eukaryotic cells contain peroxisomes, which carry out a variety of metabolic functions depending on cell type and metabolic need. In humans, the peroxisome contains enzymes that are essential for several metabolic pathways including β-oxidation of fatty acids, amino acid catabolism, isoprenoid biosynthesis and ether-phospholipid biosynthesis (van den Bosch et al., 1992; Wanders and Tager, 1998). The importance of these peroxisomal functions is underscored by the existence of several devastating human disorders in which the import system for peroxisomal matrix proteins is impaired (the peroxisomal biogenesis disorders, PBDs) or in which there are deficiencies in single peroxisomal enzymes.

The peroxisomal biogenesis disorders include Zellweger syndrome (ZS), which is the most severe form, neonatal adrenoleukodystrophy (NALD), infantile refsum disease (IRD) and rhizomelic type chondrodysplasia punctata (RCDP). PBDs are inherited in an autosomal recessive fashion, and occur with incidences between 1/25,000 and 1/50,000 births. The PBDs are characterized by hepatic and renal dysfunction, developmental delay and neurological abnormalities. Interestingly, ZS, NALD and IRD appear to represent a continuum of related diseases that all show essentially the same biochemical manifestations, including increased levels of very-long-chain fatty acids (C24:0 and C26:0), bile acid intermediates and phytanic acid (Moser, 1999), whereas RCDP patients display a distinct phenotype.

The PBDs have been assigned to 12 genetic complementation groups with most genes responsible for these disorders identified and implicated in the import of peroxisomal proteins from the cytosol to the peroxisomal matrix. The first mammalian peroxisome assembly gene was isolated using functional complementation cloning with peroxisome-deficient CHO cells (Tsukamoto et al., 1991). In addition, several human *PEX* genes have been identified on the basis of sequence comparison to yeast peroxisome biogenesis genes. The proteins they encode are now all termed peroxins (Distel et al., 1996). In humans, at least 13 peroxins are required for normal peroxisome biogenesis (Fujiki, 2000; Gould and Valle, 2000). The relationship between the biochemical abnormalities and the resulting clinical and pathological manifestations of the PBDs can be variable and is not well understood (Moser, 1999; Wanders, 1999).

The steps in peroxisome biogenesis have been elucidated on the basis of work in both yeast and human cells (Sacksteder and Gould, 2000). Proteins destined for the peroxisomal matrix are translated in the cytosol and are recognized by one of two intracellular receptors, Pex5p or Pex7p (Hettema et al., 1999;

Subramani et al., 2000). These receptors recognize proteins that contain either a C-terminal tripeptide (peroxisomal targeting signal 1, PTS1) or an N-terminal nonapeptide (peroxisomal targeting signal 2, PTS2), respectively. Pex5p contains tetratricopeptide repeat (TPR) domains thought to mediate protein–protein interactions with the PTS1 signal of matrix proteins (Gatto et al., 2000). Defects in PEX5 are associated with complementation group 2 of the PBDs (Dodt et al., 1995; Wiemer et al., 1995). Pex7p is a member of the WD-40 protein family that contains 6WD repeats. Defects in PEX7 result in rhizomelic chondrodysplasia punctata (complementation group 11) (Braverman et al., 1997).

After receptor–cargo binding, the complex docks at the peroxisomal membrane. Pex13p and Pex14p are membrane peroxins that probably serve as docking targets for the PTS1 receptor Pex5p. There are reports suggesting that Pex3p and Pex17p are also part of the docking event at the peroxisomal membrane (Huhse et al., 1998; Subramani et al., 2000). The receptor–cargo complex is then translocated into the peroxisome by a process that is poorly understood (Dammai and Subramani, 2001). The integral peroxisomal membrane proteins Pex2p, Pex10p and Pex12p are all zinc-binding ring-finger proteins that act downstream of the docking step, although their specific functions have not been clearly defined.

Our interest is in understanding the functions of peroxisomes in *Caenorhabditis elegans* and in establishing an invertebrate animal model system for the PBDs. In both plants and yeasts, the β-oxidation of fatty acids occurs solely in the peroxisome. In humans, the oxidation of short- and medium-chain fatty acids is accomplished in the mitochondria, whereas the oxidation of very-long-chain fatty acids (C:24 or longer) is carried out in the peroxisome. Bioinformatic analysis of the β-oxidation enzymes identified in the *C. elegans* genome predicts that, like humans, the nematode has both mitochondrial and peroxisomal β-oxidation pathways (Gurvitz et al., 2000), and suggests that the nematode should be a relevant system in which to model the human peroxisomal disorders. More specifically, establishing a peroxisomal biogenesis disease model in *C. elegans* should allow genetic suppressor screens that might suggest novel intervention strategies for the tragic PBDs.

The *C. elegans* genome encodes homologs of 11 of the 13 human peroxins. We found that inactivation of the *C. elegans* homologs of Pex5p, Pex6p, Pex12p, Pex13p and Pex19p by RNA-mediated interference (RNAi) produces an early larval developmental arrest before postembryonic cell division, indicating that peroxisome function is necessary for the normal development of *C. elegans*. We further show that the peroxisomal import machinery is disrupted when the *C. elegans* homolog of Pex5p, *prx*-5, is knocked down by RNAi strategies. Nematodes lacking peroxisomes exhibit a developmental block similar to starvation-arrested nematodes, although certain morphological features are different in starved nematodes. We discuss implications for roles that peroxisomes may play in postembryonic development, and the potential of this system to address human disease mechanisms.

Materials and Methods

Vectors and constructs

Primers were designed to PCR amplify a large exon (over 300 bp) from each targeted gene. For the PEX5 homolog, C34C6.6, exon 6 of this gene was amplified. For the PEX6 homolog, F39G3.7, exon 4 was amplified. Exon 4 of the PEX12 homolog F08B12.2 was amplified. For the PEX13 homolog, F32A5.6, exon 4 was amplified. For the PEX19 homolog, F54F2.8, exon 2 was amplified. PCR products were ligated into either pBluescript, or pCRII using the TOPO TA kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Recombinant plasmids containing the inserts were linearized by restriction enzyme digestion. 1-2 μg of linearized plasmid was used for in vitro transcription using the Promega Megascript kit, scaling down the reaction components to be appropriate for the amount of template. RNA was purified either by ethanol precipitation or over a RNeasy column (Qiagen, Valencia, CA). The sense and antisense RNA transcripts were checked by gel electrophoresis and mixed in approximate equimolar amounts in RNA injection buffer (20 mM KPO_4, pH 7.5, 3 mM KCitrate, pH 7.5, 2% polyethyleneglycol 6000). The RNA was annealed by heating to 65°C for 20 minutes followed by incubation at 37°C for 30 minutes. The formation of double-stranded RNA was checked by gel electrophoresis. Before loading needles for injection, the annealed dsRNA was microcentrifuged for 10 minutes.

Strains and genetic analysis

C. elegans strains were maintained at 20°C as described previously (Brenner, 1974). The N2 Bristol strain was used for all experiments unless otherwise noted. Worms were fed bacterial strain, OP50. The strain LT484 containing *Is[*p_{rnr-1}*GFP*; *rol-6(su1006)*] was kindly provided by the Padgett Lab (Waksman Institute, Rutgers University, Piscataway, NJ). The *unc-129::GFP* strain was kindly provided by the Culotti lab (University of Toronto, Ontario, Canada). The *mig-2::GFP* strain was kindly provided by the Kenyon Lab (University of California, San Francisco, CA).

Construction and analysis of GFP–SKL reporter

We generated the peroxisome-targeted GFP construct $p_{HSP16/2}$GFP–SKL by subcloning a C-terminal fragment (*Mun*I-*Xba*I) of a PTS1-tagged GFP from the vector pcDNA3-PTS1GFP (gift of S. Gould, Johns Hopkins University, Baltimore, MD). This construct has the amino acids PLHSKL added in frame to the C-terminus of GFP. This C-terminal fragment of GFP was substituted into the C-terminal region of GFP in the *C. elegans* vector pPD99.44 (gift of A. Fire, Carnegie Institution of Washington, Baltimore, MD). This vector contains the heat-shock promoter 16/2 driving expression of a GFP that contains five artificial *C. elegans* introns. Germline transformation was performed as previously described (Driscoll, 1995; Mello and Fire, 1995). Plasmids for transformation were prepared using a Qiagen miniprep kit and were injected at a concentration of 50-100 ng/μl. The transformation marker pRF4 containing *rol-6(su1006)* was co-injected at a concentration of 50 ng/μl. Transgenic rolling animals were selected among the progeny. Transgenic lines were isolated and heat-shock experiments were performed at 35°C for 4 hours for adult worms and for 1 hour for younger worms. Worms were recovered for 1-2 hours before microscopy. Worms were mounted on 2% agarose pads in M9 for fluorescence microscopy on a Zeiss Axioplan 2 microscope.

RNA interference assays

Injection of dsRNA was carried out as previously described (Fire et al., 1998). Briefly, approximately 200 ng of double-stranded RNA (dsRNA) were injected into the gonad of an L4-young adult worm. Injected worms were recovered for 8-16 hours before being moved to individual plates to enable unaffected embryos to be cleared from the hermaphrodite. The injected nematodes were separated to individual plates and transferred at either 12 or 24 hour intervals to stage development of their progeny. Development of the progeny was monitored daily and compared with noninjected time-matched control

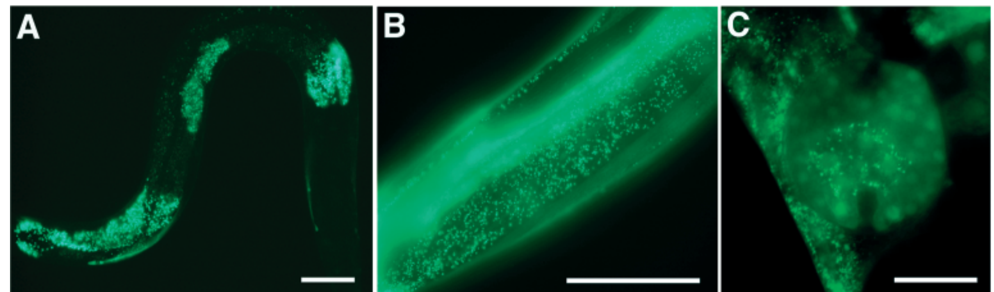


Fig. 1. GFP containing a PTS1 motif is imported into peroxisomes. Wild-type nematodes were injected with the construct p*HSP16/2*GFP–SKL to create the line, Ex[p*HSP-16/2*GFP–SKL;pRF4*(rol-6su1006)*]. Transgenic animals were heat-shocked for 4 hours at 35°C and recovered at 20°C for 2 hours before photography. A distinctly punctate pattern was observed in intestinal cells (A), in hypodermal cells (B) and in developing embryos (C). Note that for photography we selected genetically mosaic animals in which only a few cells harbored the transgene.

Table 1. *C. elegans* homologs of the mammalian peroxins

Peroxin	Proposed function*	*C. elegans* homolog	Homology (%)	E value
Pex1p	Matrix import/vesicle fusion	C11H1.6/*prx-1*	72	–61
Pex2p	Matrix protein import	ZK809.7/*prx-2*	44	–10
Pex3p	Peroxisomal membrane protein import	C15H9.8/*prx-3*	53	–14
Pex5p	PTS1 receptor	C34C6.6/*prx-5*	49	–66
Pex6p	Matrix import; vesicle fusion	F39G3.7/*prx-6*	56	–67
Pex7p	PTS2 receptor	NA		
Pex10p	Translocation machinery	C34E10.4/*wrs-2*†	40	–19
Pex11p	Proliferation/medium chain fatty acid oxidation	C47B2.8/*prx-11*‡		
Pex12p	Translocation machinery	F08B12.2/*prx-12*	56	–50
Pex13p	Docking of receptors	F32A5.6/*prx-13*	56	–42
Pex14p	Docking of receptors	R07H5.1/*prx-14*	38	–8
Pex16p	Peroxisomal membrane biogenesis	NA		
Pex19p	Peroxisomal membrane protein import	F54F2.8/*prx-19*	45	–19

*Proposed functions listed in the table are as described previously (Purdue and Lazarow, 2001). †Possible misprediction in current wormbase release. ‡Identified in Lie et al., 2002. NA, none apparent.

worms. Arrested worms were mounted in M9 buffer on 2% agarose pads for microscopy.

Results

Visualization of peroxisomes in *C. elegans*

To visualize *C. elegans* peroxisomes and to confirm a functional peroxisomal import mechanism, we constructed a gene encoding a GFP protein with a C-terminal SKL peroxisomal targeting signal. We expressed this peroxisome-targeted protein under control of the heat-shock promoter HSP-16/2 (Stringham et al., 1992). This transgene was carried as an extragenic array that can be lost during cell division to generate genetically mosaic animals in which only some cells have the transgene, facilitating cell identification.

We heat-shocked adult transgenic animals for 4 hours and characterized the GFP location after 2 hours of recovery. Peroxisomal structures have previously been identified in the intestine by GFP tagging of catalase 2 (*ctl-2*) and by immunochemical staining (Taub et al., 1999; Togo et al., 2000). In addition to clear punctate signals in the intestinal cells (Fig. 1A), we observed numerous peroxisomes in the bands of hypodermal cells that run longitudinally over the surface of the nematode (Fig. 1B). We also observed fluorescent GFP-containing particles in the developing embryos (Fig. 1C). Note that since this heat-shock promoter is not active in all cell types, our results do not exclude the existence of peroxisomes in other tissues such as neuronal cells. Our results and the results of other groups with targeting GFP to the peroxisome prove that *C. elegans* contains functional peroxisomal import machinery that is dependent on SKL (Motley et al., 2000; Taub et al., 1999). These results extend characterization of the remarkable conservation of peroxisome biogenesis to include the nematode *C. elegans*.

C. elegans peroxin homologs

Table 1 lists the human peroxins, their proposed functions and the *C. elegans* homologs, along with the statistical significance of the match expressed as E values. Because the gene classification of PEX has already been claimed in the *C. elegans* field by *P*achytene *ex*it genes, the curator of the *C. elegans* Genetic Consortium has supported the use of PRX to define the peroxisome biogenesis genes in the nematode (J. Hodgkin, personal communication). The homologs for PEX1 (*prx-1/*C11H1.6) and PEX6 (*prx-6/*F39G3.7) were also identified by Ghenea et al. and shown to be expressed mainly in intestinal cells (Ghenea et al., 2001). The homolog for PEX5 (*prx-5/*C34C6.6) binds to the peroxisomal targeting signal 1 in a yeast two-hybrid system, supporting its designation as the PEX5 homolog (Gurvitz et al., 2000). The *C. elegans* homologs for PEX2, PEX12, PEX13 and PEX19 were also identified by Petriv et al. (Petriv et al., 2002). The *C. elegans* homologs of PEX1, PEX5, PEX6, PEX12 (*prx-12/*F08B12.2) and PEX13 (*prx-13*/F32A5.6) show the greatest sequence conservation, which is more than 50% similar over the entire

protein length of each when compared with the human proteins. The PEX2 (*prx-2*/ZK809.7), PEX3 (*prx-3*/C15H9.8), PEX14 (*prx-14*/R07H5.1) and PEX10 *(prx-10/C34E10.4*) homologs are not as conserved, but are nevertheless statistically significant. The PEX11 (*prx-11/C47B2.8)* homolog was recently identified by Li et al. (Li et al., 2002).

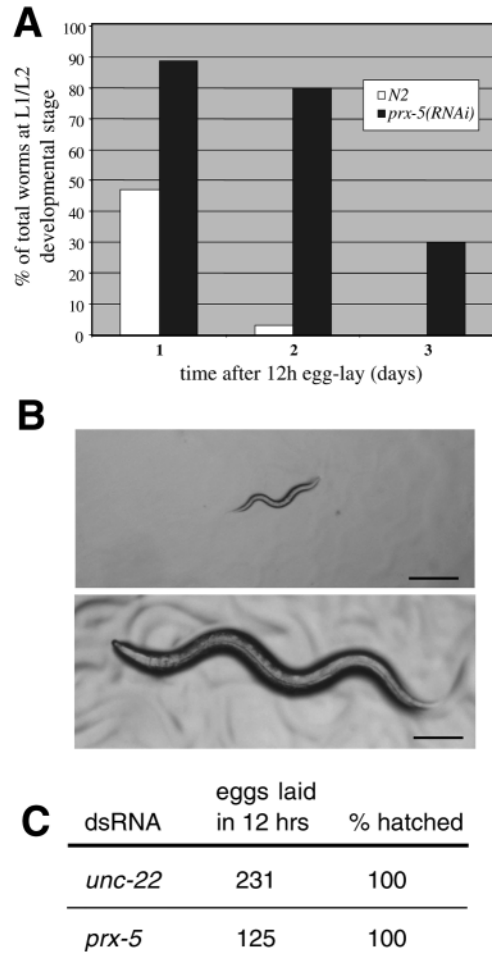
The human peroxins for which neither *C. elegans* nor *Caenorhabditis briggsae* homologs could be identified are PEX7 (the PTS2 receptor) and PEX16, which is a peroxisomal membrane protein required for membrane biogenesis (Eitzen et al., 1995). It is not surprising that we did not identify a PEX-7 homolog, given that *C. elegans* orthologs of PTS-2-containing proteins have no detectable PTS-2 signal, but instead have acquired a PTS-1 signal (de Vet et al., 1998; Motley et al., 2000). We also searched the entire *C. elegans* genome for any predicted open reading frames containing a PTS-2 motif and found none. In addition, PEX5 is expressed as a short form, lacking the PEX7 interaction motif that had been identified in the long form of PEX5 from mammals (Dodt et al., 2001).

There are ten additional peroxins that have only been isolated in one or more yeast species and we did not identify homologs of any of these peroxins, even using less-stringent search parameters. Thus, this survey indicates another aspect in which *C. elegans* peroxisomes seem to be more similar to the human than to yeast peroxisomes.

C. elegans peroxins are required for normal progression to postembryonic development

We used dsRNA interference to evaluate the biological requirements for five putative *C. elegans* peroxin genes. We targeted the *C. elegans* homologs of the PTS1 receptor Pex5p, ATPase Pex6p, peroxisomal membrane proteins Pex12p and Pex13p, and a cytosolic protein required for membrane protein import, Pex19p. We injected dsRNA into young adults and scored the progeny of the injected animals. dsRNA interference directed against these five *C. elegans* peroxins all resulted in the same phenotype – arrest at the first larval stage of development. This larval stage normally lasts about 12 hours at 20°C (Byerly et al., 1976). The arrested worms were viable and mobile, moving on the plate and through the food as would be normal for an L1 worm. The length of the larval arrest varied from 2 to 8 days (Fig. 2A). The variation in the length of arrest is probably a reflection of the efficiency of dsRNA interference and probably relates to the quantity or stability of the dsRNA introduced. Interestingly, arrested nematodes that recovered (most likely a result of the eventual degradation of the dsRNA) resume development that occurs at a normal rate and appears to have a wild-type outcome. This suggests that absence of peroxisomal function can be tolerated at least for several days in arrested L1 larvae.

Because we observed similar phenotypes in all RNAi experiments, we chose to analyze the PTS1 receptor homolog *prx-5/*C34C6.6 in more detail. Fig. 2B shows an arrested *prx-5(RNAi)* nematode 4 days after hatching, and illustrates how severely the development is arrested compared with a time-matched wild-type nematode. In addition, we observed decreased brood size in the worms injected with peroxin dsRNA. The brood size of *prx-5(RNAi)* worms was about half that of an *unc-22(RNAi)* control (Fig. 2C). Eggs that were laid, however, hatched efficiently.



dsRNA	eggs laid in 12 hrs	% hatched
unc-22	231	100
prx-5	125	100

Fig. 2. dsRNA interference directed against the *C. elegans* PEX-5 homolog C36C6.6, *prx-5*, results in larval arrest. Young adult hermaphrodites injected with dsRNA targeted to exon 6 of *prx-5* laid eggs for a 12 hour period, 16 hours after injection. (A) RNAi knockdown of *prx-5* results in developmental arrest at the L1/L2 stage. (B) Identical magnification of a *prx-5(RNAi)* nematode 3 days after being laid, and a time-matched wild-type worm. Bar, 10 µm. (C) After a 16 hour recovery period postinjection, ten viable injected worms were placed on a plate for 12 hours to measure egg production. Egg production is reduced in *prx-5(RNAi)* treated animals as compared to an *unc-22(RNAi)* control injection.

Data from genome-wide RNAi feeding and soaking experiments support our findings. The chromosome III RNAi screen undertaken by Gonczy and colleagues included both the Pex10p homolog that we did not target, and the Pex19p homolog, which we did test (Gonczy et al., 2000). In the case of the Pex19p homolog, a similar phenotype, arrested development, was reported and, in the case of the Pex10p homolog, a Gro phenotype of slowed development was scored. Maeda and colleagues reported that targeting of F39G3.7, the Pex6p homolog, causes a sick phenotype in their large-scale RNAi screen (Maeda et al., 2001). In both cases, the broad scope of the screens permitted examination of only gross phenotypes, which were not pursued in detail. Recently, Petriv et al. found that RNAi inactivation of *prx-5, prx-12, prx-13* and *prx-19* resulted in a developmental delay and a greatly reduced

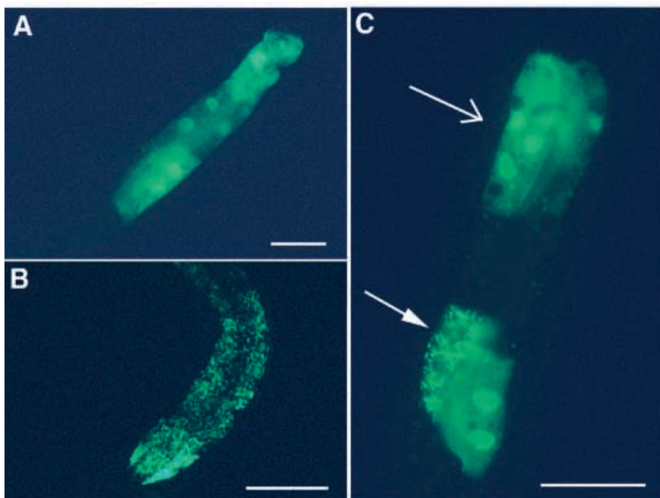


Fig. 3. Peroxisomal import is defective in *prx-5(RNAi)* nematodes. Ex[p$_{HSP16/2}$GFP-SKL] nematodes were injected with dsRNA targeted to exon 6 of *prx-5*. Progeny were heat-shocked for 2 hours at 30°C and recovered for 1 hour. A disruption of peroxisomal localization of the GFP–SKL is seen in (A), which results in the GFP remaining cytosolic when compared with a control nematode in (B). In some animals we observed a mosaic of GFP subcellular localization, in that both cells with cytosolic and punctate GFP were observed in the same animal (C). Cells with GFP in all panels are intestinal cells. Bars, 5 μm.

percentage of adult progeny 3 days following injection of dsRNA homologous to these genes (Petriv et al., 2002). The authors were not able to specify whether this was due to delayed development or to an arrest in development. Our results allow us to conclude that lack of any of these peroxins results in a developmental arrest at the L1 stage. Furthermore, Petriv et al. did not find developmental delay when targeting dsRNA to the PEX-6 homolog, *prx-6* (Petriv et al., 2002).

prx-5 is required for peroxisomal import

We injected *prx-5* dsRNA into animals harboring the p$_{HSP16-2}$GFP–SKL;pRF4 extragenic array. When we heat-shocked the progeny of these animals we noted that the GFP–SKL was no longer localized in punctate structures in the cells that expressed GFP. Instead, the animals had GFP–SKL distributed through the cytoplasm and nucleus (see Fig. 3A,B, which shows the intestinal cells of injected vs. a noninjected control animal). In a few animals we were able to detect two classes of cells – those that show punctate GFP localization and those that have diffuse and nuclear GFP probably due to incomplete RNAi effects (see Fig. 3C). We conclude that *prx-5* is required for peroxisomal import of proteins containing the C-terminal targeting signal SKL. Importantly, the phenotype of failure to import SKL proteins to the peroxisomes is exactly the phenotype in human cell lines from patients with peroxisomal disorders (Santos et al., 1988).

prx-5(RNAi)-arrested nematodes do not initiate postembryonic development

We injected *prx-5* dsRNA into strain LT483 that contains an integrated GFP reporter under control of the ribonucleotide reductase (*rnr-1*) gene promoter. This promoter is active only during the S phase of the cell cycle and expression of GFP can be considered a marker for dividing nuclei. After an embryo hatches and begins postembryonic development, a series of invariant cell divisions occur (Sulston and Horvitz, 1977). In the p$_{rnr-1}$GFP strain these cells in the L1 animal become labeled by GFP (see Fig. 4E for an early L1 animal and Fig.

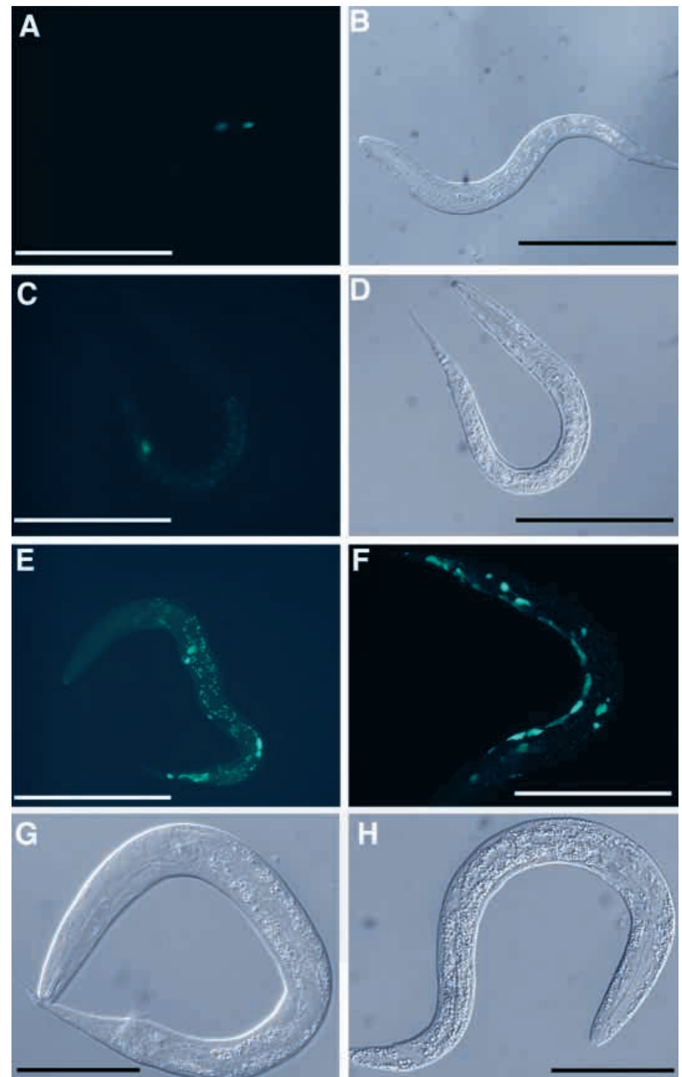


Fig. 4. *prx-5(RNAi)*-arrested nematodes do not initiate postembryonic cell divisions. Strain LT483 contains an integrated 2X *rnr-1* promoter driving expression of GFP. We injected this strain with dsRNA targeted to *prx-5* and examined the progeny. The animal in A and B has just two faint cells that contain GFP. Eggs from this strain were hatched in the absence of food to induce a post-hatching starvation-arrest (C,D). *prx-5(RNAi)* animals and starvation-arrested animals of this strain have similar GFP expression patterns, and in addition they lack autofluorescence in their guts (A,C). (E) A young p$_{rnr-1}$GFP L1 animal in which divided nuclei are apparent by their more robust GFP signal. (F) An older p$_{rnr-1}$GFP L1 animal that contains numerous dividing nuclei. (G) A 4 day arrested *prx-5(RNAi).* This animal has been arrested for 4 days, and does not contain the numerous refractile structures found in a 4-day old starvation-arrested worm (H). Bars, 10 μm.

4F for an older L1 animal). $p_{rnr\text{-}1}$GFP;*prx-5(RNAi)* animals display an abnormal pattern of GFP expression with at most one or two cells in each animal labeled by GFP (Fig. 4A,B). To determine whether these GFP-labeled cells marked by GFP result from postembryonic cell divisions, rather than remnants of undegraded GFP from embryonic development, we placed eggs onto plates without food to induce a starvation arrest of the hatched animals. When *C. elegans* embryos hatch in the absence of food they fail to initiate postembryonic development (Johnson et al., 1984). Interestingly, when we compared the $p_{rnr\text{-}1}$GFP;*prx-5(RNAi)* GFP expression pattern to starvation-arrested $p_{rnr\text{-}1}$GFP animals we also observed only one or two cells per animal that express GFP (Fig. 4C,D). Thus, in both *prx-5(RNAi)* and starvation-arrested animals, the nuclear divisions that report progression into L1 development do not occur. Furthermore, we could not detect Q-cell migration when *prx-5* dsRNA was injected into a $p_{mig\text{-}2}$GFP, nor could we see any axonal migration when *prx-5* dsRNA was injected into an UNC-129::GFP strain (Colavita et al., 1998; Honigberg and Kenyon, 2000). These are events that should occur during the L1 stage but could not be detected in *prx-5(RNAi)* animals (data not shown).

We used Nomarski optics to further examine starvation-arrested animals. Starvation-arrested worms accumulate refractile structures in the intestinal cells and throughout their bodies (Fig. 4). Although we could detect some of these refractile objects in our *prx-5(RNAi)*-arrested worms, they were not nearly as abundant and did not appear to increase significantly in number as length of arrest increased (Fig. 4). Thus, although *prx-5(RNAi)* animals are blocked at a similar stage as starvation-arrested nematodes, and are unable to initiate postembryonic development, there are morphological differences between peroxisome-deficient arrest and starvation-arrest. We conclude that peroxisomal function plays a crucial role in *C. elegans* postembryonic development and, more specifically, the absence of peroxisome function causes a very early and broad block because all postembryonic cell divisions and cell migrations we monitored did not occur.

Discussion

Peroxisomal function is required for *C. elegans* postembryonic development

The *C. elegans* genome encodes homologs for 11 of the 13 human peroxins. This conservation suggests that peroxisome biogenesis mechanisms are similar in nematodes and humans. Inactivation of five of these peroxins by RNA-mediated interference results in an early larval arrest, indicating that peroxisomal function is required for postembryonic development. The peroxisome-deficient animals do not initiate the conventional cell divisions that are hallmarks of progression through the first larval stage, which is similar to starvation-arrested animals.

Each of the peroxins we targeted by RNAi should leave the animal without functional peroxisomes and affect all biochemical pathways that would normally occur in this organelle. It is not clear at this point whether L1 progression is blocked by the presence of a general toxin or by the failure to produce a required metabolite. However, the coincidence in phenotypes of starved L1s and peroxisome-deficient animals may provide a clue as to the biochemical reason for arrest. It is proposed that in nematode food there is a molecule that acts as a signal for progression into L1 or for exit out of the dauer stage into reproductive growth (Bargmann and Horvitz, 1991). It is possible that without peroxisomal function, metabolism of this ‘signal’ molecule or one of its byproducts is disrupted, leading to a block in the pathway that signals the progression of postembryonic development.

Starvation-arrested nematodes have not been extensively studied. However, both RNA levels and enzymatic activities of the glyoxylate cycle enzymes are induced in L1-starvation arrested nematodes, as well as in embryos and dauer larvae (Khan and McFadden, 1982; Liu et al., 1997; O’Riordan and Burnell, 1990). The glyoxylate cycle enables plants and micro-organisms to synthesize carbohydrate from fatty acids (via acetyl-CoA) and the enzymes for this pathway are found in specialized peroxisomes called glyoxysomes (Cooper and Beevers, 1969; Parsons et al., 2001). The *C. elegans* enzymes of the glyoxylate cycle do not contain a PTS1 at their C-terminus, suggesting either that they may use a different pathway for import into peroxisomes or that the gyloxylate cycle is not localized in the peroxisome. The glyoxylate cycle is nevertheless dependent on the peroxisomal β-oxidation of fatty acids for a source of acetyl-CoA. In *prx-5(RNAi)* animals, the activity of the glyoxylate cycle is probably reduced due to lack of substrate. Interstingly, a peroxisomal protein comatose (CTS) in *Arabidopsis* is required for the transition from dormancy to germination and vegetative growth (Footitt et al., 2002). The CTS protein is required for lipid mobilization and transport of acyl CoAs into the peroxisome and is thus a major control point for the switch between the opposing developmental programs of dormancy and germination. It is possible that peroxisomal function is required in *C. elegans* to effect a switch over to postembryonic development in an analogous developmental control point as in *Arabidopsis* development.

C. elegans as a model system for the PBDs

In both plants and yeasts the β-oxidation of fatty acids is accomplished solely in the peroxisome. In humans there is an additional β-oxidation compartment, the mitochondria. Bioinformatic analysis of β-oxidation enzymes in *C. elegans* have provided initial evidence that nematodes are similar to humans in that some homologs of the β-oxidation enzymes contain mitochondrial signal sequences, whereas others contain a peroxisomal targeting signal. This is an indication that both cellular compartments can carry out the oxidation of fatty acids (Gurvitz et al., 2000). The striking similarity to humans suggests that *C. elegans* may be particularly well suited to addressing problems in peroxisome biochemistry relevant to the human PBDs.

The PBDs are characterized by global developmental delay and defects in neuroblast migration during development (Moser, 1999). The well-documented developmental program of *C. elegans,* coupled with its transparency and ease of culture, make the nematode an attractive organism in which to study the pathogenesis of the PBDs. Although all cell migrations and neuronal growth were blocked in the marker strains that we examined, possibly looking at migrations that occur earlier in development may shed light onto the cause of the developmental arrest. *C. elegans* is the only model system

which allows such facile observation of the nervous system in the background of disruption of peroxisome biogenesis.

In addition to the biochemical similarities, we have shown at the cellular level that disruption of *prx-5* leads to the mislocalization of a peroxisome-targeted GFP. Failure to localize peroxisome-targeted proteins is exactly the cellular phenotype found in patients in complementation group 2 of the PBDs (Dodt et al., 1995; Wiemer et al., 1995). These patients have defects in *pex*-5. The severity of the developmental arrest phenotype in *C. elegans* leads to the feasibility of development of genetic suppressor screens to overcome or circumvent the peroxisomal deficiency. Genetic suppressor analysis may shed new insight into disease mechanisms of peroxisomal biogenesis disorders and also enable us to learn about mechanisms of developmental arrest associated with peroxisomal deficiency.

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