



## Research Article

# Predicting the function and subcellular location of *Caenorhabditis elegans* proteins similar to *Saccharomyces cerevisiae* $\beta$ -oxidation enzymes

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## Abstract

The role of peroxisomal processes in the maintenance of neurons has not been thoroughly investigated. We propose using *Caenorhabditis elegans* as a model organism for studying the molecular basis underlying neurodegeneration in certain human peroxisomal disorders, e.g. Zellweger syndrome, since the nematode neural network is well characterized and relatively simple in function. Here we have identified *C. elegans* PEX-5 (C34C6.6) representing the receptor for peroxisomal targeting signal type 1 (PTS1), defective in patients with such disorders. PEX-5 interacted strongly in a two-hybrid assay with Gal4p-SKL, and a screen using PEX-5 identified interaction partners that were predominantly terminated with PTS1 or its variants. A list of *C. elegans* proteins with similarities to well-characterized yeast  $\beta$ -oxidation enzymes was compiled by homology probing. The possible subcellular localization of these orthologues was predicted using an algorithm based on trafficking signals. Examining the C termini of selected nematode proteins for PTS1 function substantiated predictions made regarding the proteins' peroxisomal location. It is concluded that the eukaryotic PEX5-dependent route for importing PTS1-containing proteins into peroxisomes is conserved in nematodes. *C. elegans* might emerge as an attractive model system for studying the importance of peroxisomes and affiliated processes in neurodegeneration, and also for studying a  $\beta$ -oxidation process that is potentially compartmentalized in both mitochondria and peroxisomes. Copyright © 2000 John Wiley & Sons, Ltd.

**Keywords:** *Caenorhabditis elegans*; *Saccharomyces cerevisiae*;  $\beta$ -oxidation; peroxisome; PTS1; PEX-5; tetratricopeptide repeat (TPR); two hybrid

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## Introduction

The recent completion of the nematode genome sequencing project (Consortium, 1998) promises to provide valuable insights into hitherto uncharacterized biochemical processes in *Caenorhabditis elegans* that have already been extensively studied in *Saccharomyces cerevisiae* (Chervitz *et al.*, 1998), such as  $\beta$ -oxidation of fatty acids. The  $\beta$ -oxidation process occurs in the peroxisomal compartment of all eukaryotic systems tested and, additionally, in the mitochondria of mammalian cells (Kunau *et al.*, 1995). Peroxisomes are organelles demarcated by a

single membrane that characteristically contain enzymes for oxidative reactions involving molecular oxygen and for metabolizing hydrogen peroxide (de Duve and Baudhuin, 1966).

Peroxisomal matrix enzymes, including those involved in  $\beta$ -oxidation, are imported with the help of specific targeting signals that interact with cognate receptors. The type 1 peroxisomal targeting signal (PTS1) of some proteins is a carboxy-terminal tripeptide that consists of SKL or a variant thereof (Gould *et al.*, 1987; Subramani, 1993), i.e. S/A/C-K/R/H-L (McNew and Goodman, 1996). PTS1 is recognized by Pex5p (Van der Leij *et al.*,

1993) in all organisms studied to date. Pex5p has been shown to accommodate considerable degeneracy at the PTS1 (Sommer *et al.*, 1992; Elgersma *et al.*, 1996; Kragler *et al.*, 1998), and to depend on additional residues adjacent to the C-terminal tripeptide for its binding affinity to cargo protein (Lametschwandtner *et al.*, 1998). A second peroxisomal protein targeting signal, PTS2, consists of the sequence R/K-L/I-X<sub>5</sub>-H/Q-L (McNew and Goodman, 1996). The PTS2 receptor is represented by Pex7p (Marzioch *et al.*, 1994; Rehling *et al.*, 1996). PTS2 is cleaved off in some instances, e.g. rat thiolase and watermelon malate dehydrogenase, but not in others, e.g. yeast thiolase and amine oxidase (Subramani, 1993). Some peroxisomal matrix enzymes do not contain either of the two known targeting signals (Karpichev and Small, 2000).

Peroxisomal diseases in humans often lead to degeneration or poor development of neurons. For example, Zellweger syndrome representing a class of peroxisome biogenesis disorders in which the entire peroxisomal compartment is compromised is associated with severe neurological impairments; PEX5 is defective in a number of Zellweger patients (Online Mendelian Inheritance in Man, OMIM<sup>TM</sup>, Johns Hopkins University, Baltimore, MD; <http://www.ncbi.nlm.nih.gov/omim/>, OMIM number 214100: 6/4/1998). Neurological abnormalities can also be caused by single peroxisomal  $\beta$ -oxidation enzyme deficiencies, such as peroxisomal 3-oxoacyl-CoA thiolase deficiency (OMIM number 261510: 4/25/2000), peroxisomal L-bifunctional protein (OMIM number 261515: 3/30/1999) and peroxisomal D-bifunctional protein (OMIM number 601860: 9/30/1999; Itoh *et al.*, 2000). Since the neural development of *C. elegans* has been characterized (Sulston *et al.*, 1983), the nematode might serve as a model organism for studying the possible roles of peroxisomes and the biochemical processes contained therein in the development and maintenance of neurons.

A further reason for considering nematodes as a better alternative to fungal models for investigating fatty acid degradation lies in the subcellular distribution of  $\beta$ -oxidation. In fungi,  $\beta$ -oxidation is strictly a peroxisomal process (Kunau *et al.*, 1995). Nematodes might turn out to resemble mammals by possessing both peroxisomal and mitochondrial  $\beta$ -oxidation, and could therefore serve as a more appropriate model system than *S. cerevisiae* for studying the relative contribution of the two

compartments to fatty acid breakdown. *C. elegans* peroxisomes have been shown previously to contain catalase (Maebuchi *et al.*, 1999; Togo *et al.*, 2000), an enzyme intimately associated with H<sub>2</sub>O<sub>2</sub>-generating enzymes, among them acyl-CoA oxidase, involved in peroxisomal fatty acid  $\beta$ -oxidation. However, it is hitherto unclear whether: (a) the conjectured *C. elegans*  $\beta$ -oxidation process is compartmentalized in peroxisomes; and (b) as in mammals, the process hypothesized to occur in nematode peroxisomes is augmented by a mitochondrial one.

In this study we considered *C. elegans* as a model system for investigating the possible connection between peroxisomal processes and neuronal development. To assess whether nematode peroxisomes potentially accommodate  $\beta$ -oxidation enzymes, the *C. elegans* databases were searched for putative orthologues of the PTS1 receptor, and for proteins similar to *S. cerevisiae* enzymes with  $\beta$ -oxidation functions. By additionally searching for the presence of PTS1, PTS2, and mitochondrial leader sequences in these nematode proteins using the PSORT II algorithm, a list was compiled representing candidate peroxisomal and mitochondrial  $\beta$ -oxidation enzymes. To underscore the algorithm predictions as to which of the nematode proteins could represent a peroxisomal protein due to a potential PTS1, *C. elegans* PEX-5 was used to analyse C-terminal tripeptides occurring on selected nematode proteins in two-hybrid interaction assays.

## Materials and methods

### Strains, plasmids and transformations

*Escherichia coli* strain DH10B was used for all plasmid amplifications and isolations. The *S. cerevisiae* strains and plasmids used here are described in Table 1. The yeast two-hybrid strain PCY3 is a derivative of PCY2 (Chevray and Nathans, 1992). Transformations of yeast strains were performed according to Chen *et al.* (1992).

### Growth conditions

Yeast transformants were selected on minimal medium (SC) containing 0.67% (w/v) Yeast Nitrogen Base without amino acids, 2% (w/v) D-glucose, 2% (w/v) agar, and supplemented with bases and amino acids (20–150  $\mu$ g/ml) as required

(SC–Leu–Trp). For quantitative measurements of  $\beta$ -galactosidase activity, yeast cells were grown overnight at 30°C with shaking in liquid selective medium (as above but without agar), diluted to  $A_{600}=0.2$  in the same medium, and collected after 6–8 h at  $A_{600}=1$ .

### Computer-based predictions

The amino acid sequences of *S. cerevisiae* peroxisomal proteins were obtained from YPD<sup>TM</sup> (Costanzo *et al.*, 2000), and compared using the BLAST programme (Altschul *et al.*, 1997) with the worm database WormPD<sup>TM</sup> (<http://www.proteome.com/databases/index.html>). Each nematode protein was then analysed using the PSORT II programme for predicting the subcellular sites of proteins based on their amino acid sequences (<http://www2.imcb.osaka-u.ac.jp/psort/form2.html>). The peroxisomal matrix-targeting sequences used by the PSORT II programme were based on the published consensus for PTS1 and PTS2 (McNew and Goodman, 1996), as noted in the Introduction. For predicting mitochondrial proteins, PSORT II employed a method that recognizes mitochondrial targeting signals based on the amino acid composition of the N-terminal 20 residues. The PSORT II users' manual does not specify the algorithm's reasoning system in a situation in which a protein could contain both a mitochondrial and a peroxisomal targeting signal. The values in the tables represent the percentage probability for any given protein to be located in a particular subcellular site.

### Molecular cloning of *C. elegans* *pex-5*

The oligonucleotides used here are listed in Table 1. General nucleic acid manipulations were done according to standard protocols (Sambrook *et al.*, 1989). Purification of DNA following agarose electrophoresis was performed using QIAEX II (Qiagen Inc., Valencia, CA). Nematode cDNA was made by applying reverse transcription to purified nematode RNA using the 3'-end oligonucleotide H514, according to the manufacturer's protocol. To obtain the cDNA for the complete nematode PEX-5 (502 amino acids), polymerase chain reaction (PCR) was applied to cDNA obtained from the previous reverse transcription reaction using 50 pmol each of oligonucleotides H513 and H514 at an annealing temperature of 50°C (25 cycles) and a 1 : 9 mixture of *Pfu* and *Taq* DNA polymerases. This resulted in a

single 1.5 kb DNA fragment that was digested with *KpnI* (cutting within the nucleotide sequence of H513) and *SphI* (cutting within H514). The digested DNA was ligated into a similarly digested plasmid vector pUC18, resulting in plasmid pSL5. Automatic nucleotide sequencing verified that no mutations were introduced into the PCR product.

To obtain the C-terminal half (291 amino acids) of nematode PEX-5, a similar approach was followed using oligonucleotides H512 and H514. An *EcoRI*- and *SphI*-doubly digested PCR product was ligated to a similarly cut pUC18, resulting in plasmid pSL2. Nucleotide sequencing revealed a TTA to TTG wobble mutation at the Leu codon representing amino acid position 306 in PEX-5. To generate a two-hybrid construct encoding the tetratricopeptide repeat domain of PEX-5 in pGBT9 (Bartel *et al.*, 1993), PCR was applied to pSL2 template DNA using oligonucleotides H542 and H543. The resulting 0.8 kb PCR product encoding 267 amino acids of the PEX-5 C terminus (amino acid positions 215–481) was digested with *EcoRI* and *BamHI* and inserted into a similarly digested pGBT9, resulting in pSL8. The insert was verified by automatic sequencing.

### Two-hybrid screen and enzyme assays

Two-hybrid experiments were conducted as described (Lametschwandtner *et al.*, 1998) according to the Matchmaker two-hybrid system protocol (Clontech Laboratories, Inc., Palo Alto, CA). A pSL8-containing yeast strain PCY3 (ySL1) was additionally transformed using a pGAD GH-derived peptide library consisting of 16 NN(T/G) codons fused to the activation domain of yeast Gal4p (Yang *et al.*, 1995). Transformants were replica-plated to selective medium containing 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), and blue colonies were isolated for further analysis. Library plasmids from blue single colonies were isolated and verified by reintroduction to ySL1 cells.  $\beta$ -Galactosidase activity was confirmed on X-gal plates, and the corresponding library plasmids sequenced. For two-hybrid interactions,  $\beta$ -galactosidase activity measurements were conducted using *O*-nitrophenyl- $\beta$ -D-galactoside, as described (Miller, 1972). Protein concentrations were determined according to the method of Bradford (1976). Values reported here represent the mean of three independent experiments per strain,  $\pm$  standard deviation.

Table 1. *S. cerevisiae* strains, plasmids and oligonucleotides used

Strain, plasmid or oligonucleotide	Description	Source or reference
<i>Strains</i> <sup>a</sup>		
(1) PCY3	<i>MAT<math>\alpha</math> his3-<math>\Delta</math>200 ade2-101<sup>ochre</sup> trp1-<math>\Delta</math>63 leu2 gal4<math>\Delta</math> gal80<math>\Delta</math> LYS2::GALI-HIS3 URA3::GALI-lacZ</i>	(Chevray and Nathans, 1992)
(2) yGLI <sup>1</sup>	Containing plasmid pAH987	(Lametschwandtner et al., 1998)
(3) ySLI <sup>1</sup>	Containing plasmid pSL8	This study
yAG1254 <sup>2</sup>	Containing plasmid Sc01	This study
yAG1255 <sup>2</sup>	Containing plasmid PLI_4	This study
yAG1256 <sup>3</sup>	Containing plasmid Sc01	This study
yAG1257 <sup>3</sup>	Containing plasmid PLI_4	This study
yAG1262 <sup>3</sup>	Containing plasmid Sc07	This study
yAG1263 <sup>3</sup>	Containing plasmid Sc08	This study
yAG1264 <sup>3</sup>	Containing plasmid Sc09	This study
yAG1265 <sup>3</sup>	Containing plasmid Sc10	This study
yAG1266 <sup>3</sup>	Containing plasmid Sc18	This study
yAG1267 <sup>3</sup>	Containing plasmid Sc22	This study
yAG1268 <sup>3</sup>	Containing plasmid Sc24	This study
yAG1269 <sup>3</sup>	Containing plasmid Hs40	This study
yAG1270 <sup>3</sup>	Containing plasmid Sc26	This study
yAG1271 <sup>3</sup>	Containing plasmid Sc27	This study
<i>Plasmids</i>		
pSL5	cDNA for the complete <i>C. elegans</i> PEX-5 (502 aa) in pUC18	This study
pSL2	cDNA for the C-terminus of <i>C. elegans</i> PEX-5 (291 aa) in pUC18	This study
pSL8	pGBT9 containing the cDNA for <i>C. elegans</i> PEX-5 TPR domain (267 aa)	This study
pAH987	pGBT9 containing the DNA for <i>S. cerevisiae</i> Pex5p TPR domain (305 aa)	(Lametschwandtner et al., 1998)
PLI_4	pGAD GH expressing Gal4p-FE WGS QGWTRGRMHML	(Lametschwandtner et al., 1998)
Sc01	pGAD GH expressing Gal4p-CERSKL	(Lametschwandtner et al., 1998)
Sc07	pGAD GH expressing Gal4p-QRKANGRDRGGWWAKL	(Lametschwandtner et al., 1998)
Sc08	pGAD GH expressing Gal4p-RQRELSANALGGLAKL	(Lametschwandtner et al., 1998)
Sc09	pGAD GH expressing Gal4p-NGMTRSGRQGGFACL	(Lametschwandtner et al., 1998)
Sc18	pGAD GH expressing Gal4p-SGGVVARAAKM	(Lametschwandtner et al., 1998)
Sc22	pGAD GH expressing Gal4p-TWNRETGLNAVYGKL	(Lametschwandtner et al., 1998)
Sc24	pGAD GH expressing Gal4p-GKNRGSSESHGSARQKL	(Lametschwandtner et al., 1998)
Sc26	pGAD GH expressing Gal4p-KRVWRRQWSTGRKLLK	(Lametschwandtner et al., 1998)
Sc27	pGAD GH expressing Gal4p-WYGP GPCCRRRLDLK	(Lametschwandtner et al., 1998)
Hs40	pGAD GH expressing Gal4p-EGLIVMLERGLK	(Lametschwandtner et al., 1998)
pGBT9	Two-hybrid construct containing the Gal4p DNA binding domain	(Bartel et al., 1993)
pGAD424	Corresponding two-hybrid construct with the Gal4p activation domain	(Bartel et al., 1993)
<i>Oligonucleotides</i>		
H512	5'-AGGAGAATTCGCAAACCCCTTTCACAACCATGTCAG-3'	This study
H513	5'-AGGAGAGGTACCATGAAAGGAGTTGTAGAAGGACAATG-3'	This study
H514	5'-GAAGAGGCATGCGGATCCCTAGACTAGAGAGGCTTTTACAGC-3'	This study
H542	5'-AGGAGAATTCGACAGGTAGCGGTAGCAATTTTACAACCATGTCAG ATCCAC-3'	This study
H543	5'-GAAGAGGCATGCGGATCCCTAGACATTCGATGTTTCGGATTGCTGC-3'	This study

<sup>a</sup>The numbers in superscript following the strains' designations refer to their parental genotypes, e.g. yGLI<sup>1</sup> was derived from (1) PCY3. aa = amino acids. TPR = tetratricopeptide repeat

## Results

### Identification of *C. elegans* PEX-5

Import of most yeast proteins into the peroxisomal matrix depends on Pex5p (Van der Leij et al., 1993).

One of the basic criteria for the predictions made in the course of this study using the PSORT II algorithm was the potential presence or absence of the cognate signal PTS1 in candidate  $\beta$ -oxidation enzymes. The consensus for PTS1 is no longer

precise since non-canonical C-terminal tripeptides can function as PTS1 due to degeneracy and to neighbouring amino acid residues acting as PTS1 enhancers (Elgersma *et al.*, 1996; Lametschwandtner *et al.*, 1998). Hence, relying alone on an algorithm recognizing the strict consensus might exclude potential peroxisomal matrix proteins terminating with non-canonical PTS1s.

To gain a better understanding of whether proteins predicted by computer algorithms to contain PTS1s potentially represented peroxisomal enzymes, the C termini of such candidates could be examined for PTS1 function. This could be done by assaying for interactions with the cognate Pex5p, provided of course that the mechanism of protein import is conserved in the organism under investigation. For example, previous yeast two-hybrid experiments using *S. cerevisiae* Pex5p predicted that a non-conserved C-terminal tripeptide HRL might function as PTS1 (Lametschwandtner *et al.*, 1998). This prediction was subsequently validated by the discovery of a yeast peroxisomal protein Eci1p terminating with this tripeptide (Gurvitz *et al.*, 1998; Geisbrecht *et al.*, 1998), albeit the protein is proposed to contain an additional cryptic PTS (Karpichev and Small, 2000).

A search of the *C. elegans* databases for proteins with similarities to yeast Pex5p revealed a putative protein, C34C6.6, that additionally showed a strong similarity to all other known PTS1 receptors, including the most recently identified *Leishmania donovani* counterpart (Jardim *et al.*, 2000). Since yeast Pex5p (612 amino acids) was more similar to the 502 amino acid-long protein C34C6.6 (28% identity, 44% similarity over a length of 471 amino acids; WormPD<sup>TM</sup>) than to other nematode proteins with tetratricopeptide repeat motifs, this meant that C34C6.6 could represent nematode PEX-5.

To confirm the identity of C34C6.6 as PEX-5, the open reading frame for this novel protein was amplified by applying polymerase chain reaction to nematode cDNA. The nucleotide sequence of the resulting DNA fragment matched that of C34C6.6 cDNA. A fragment representing the C-terminal half of the putative PEX-5 (267 amino acids), which included these tetratricopeptide repeat motifs, was ligated to the appropriate vector for two-hybrid interaction studies, and two-hybrid assays were performed on Gal4p-activating domains extended at their respective carboxy-

termini with either SKL or HML. These and subsequent Gal4p fusion proteins used differed from one another only with respect to their C-terminal 16 (or less) amino acids (Table 1). Although these fusions presumably make available for interaction with PEX-5 additional amino acids, current knowledge stipulates that PTS1 is confined to the C termini of peroxisomal proteins. Hence, in the present analyses only the terminal three to four amino acid residues were considered.

The rationale for choosing SKL as a positive interacting partner was fairly straightforward, since it represents the canonical PTS1 in practically all organisms tested (Gould *et al.*, 1987, 1990). The C-terminal tripeptide HML, representing a less efficient PTS1, was chosen as an arbitrary control since it does not meet the strict consensus and was therefore expected to interact significantly less efficiently with the Pex5p-like protein. As a comparison, these SKL- and HML C termini were also assayed using yeast Pex5p (Table 2). Measurements of  $\beta$ -galactosidase activities demonstrated that, like the yeast PTS1 receptor, C34C6.6 also interacted with SKL to an approximately four-fold greater extent than with HML (Table 2), thereby arguing in favour of its function as nematode PEX-5.

To emphasize the function of C34C6.6 as the nematode PTS1 receptor, a two-hybrid screen was conducted using a protein library consisting of Gal4p extended by random C-termini in a similar manner to that previously performed with the yeast and human counterparts (Lametschwandtner *et al.*,

**Table 2.** Comparison of two-hybrid interactions between *C. elegans* PEX-5 or *S. cerevisiae* Pex5p and SKL- or HML-extended protein partners

Strain	C terminus	$\beta$ -Galactosidase activity <sup>a</sup>	Relative level
<i>C. elegans</i> PEX-5			
yAG1255	M-HML <sup>b</sup>	123.8 $\pm$ 24.0	1.0
yAG1254	R-SKL	556.8 $\pm$ 5.2	4.5
<i>S. cerevisiae</i> Pex5p			
yAG1257	M-HML	39.0 $\pm$ 1.6	1.0
yAG1256	R-SKL	162.6 $\pm$ 9.7	4.2

<sup>a</sup>nmol *O*-nitrophenyl- $\beta$ -D-galactoside hydrolysed/min/mg protein; mean  $\pm$  SD, *n* = 3.

<sup>b</sup>The amino acid to the left of the hyphen is adjacent to the C-terminal tripeptide and may be important for PTS1 function (Lametschwandtner *et al.*, 1998).

Table 3. C termini of Gal4p proteins interacting with *C. elegans* PEX-5

Canonical PTS1s <sup>a</sup>		Non-canonical PTS1s <sup>b</sup>		Other C-terminal tripeptides	
SKL	5	SSL	2	GKL	1
AKL	8	HKL	2	AKV	1
ARL	7	CSL	2	AAL	1
SRL	1	SML	2	AML	1
CKL	1	ASL	2	SKM	1
Total	22			15	4

<sup>a</sup>PTS1s conforming to the consensus S/A/C-K/R/H-L (McNew and Goodman, 1996).

<sup>b</sup>PTS1s not fully conforming to this consensus, but to the 'two out of three' rule (Elgersma *et al.*, 1996).

1998). Verified library plasmids obtained from a representative batch of blue single colonies were sequenced and the corresponding C-termini deduced. The results (Table 3) demonstrated that C34C6.6 interacted preferentially with canonical PTS1s (22) as well as non-canonical ones (15) compared with other C termini (4), bearing favourably on our conjecture that it represents nematode PEX-5. These results were generally very similar to those previously obtained using screens based on the human, yeast or plant PTS1 receptors (Kragler *et al.*, 1998; Lametschwandtner *et al.*, 1998), in that most of the interacting C termini turned out to be either canonical or non-canonical PTS1s. An exhaustive two-hybrid screen using *C. elegans* PEX-5 is the subject of current investigation, and is therefore not discussed further. With this PEX-5 two-hybrid construct at hand it was possible to verify experimentally the algorithm predictions made in the following sections regarding PTS1-terminating nematode proteins that could be involved in  $\beta$ -oxidation.

### *C. elegans* orthologues of classical $\beta$ -oxidation enzymes: Pox1p-like proteins

The four *S. cerevisiae* enzyme activities of 'classical'  $\beta$ -oxidation include Pox1p, acyl-CoA oxidase; Fox2p, 2-enoyl-CoA hydratase 2 and D-specific 3-hydroxyacyl-CoA dehydrogenase; and Pot1p/Fox3p, 3-ketoacyl-CoA thiolase (Dmochowska *et al.*, 1990; Hiltunen *et al.*, 1992; Igual *et al.*, 1991; Einerhand *et al.*, 1991). To identify potential Pox1p orthologues, a search of the *C. elegans* database was performed. Although *S. cerevisiae*

contains only one acyl-CoA oxidase (whose import route has not yet been identified), other yeast species contain several peroxisomal isoenzymes. For example, the yeast *Yarrowia lipolytica* contains at least five *POX* genes (Wang *et al.*, 1999).

The result of the computer-based search for Pox1p-like nematode proteins is shown in Table 4. As expected, the PSORT II algorithm predicted those worm proteins terminating with SKL to be primarily peroxisomal (Table 4). Of the remaining proteins, the AKM-ending Pox1p orthologue was predicted to be a cytosolic protein (Table 4), whereas those orthologues terminating with AKL, a canonical PTS1, were predicted to be peroxisomal or mitochondrial (although they all contain identical 15-mer carboxyl termini; WormPD<sup>TM</sup>). To test the validity of the computer-based predictions regarding the PTS1 function of these tripeptides, nematode PEX-5 was used to examine two-hybrid interactions with representative C termini extending from the Gal4p activation domain (Table 5).

The two-hybrid results demonstrated that AKL-terminating Gal4p proteins interacted with the nematode PEX-5 1.6–6.6-fold more efficiently than the protein ending with HML, whereas the AKM terminus tested was 1.7-fold more efficient than HML (upper two clusters, Table 5). This variation in interaction efficiencies among the AKL termini could have been due to specific amino acids at other positions in the proteins tested (Kragler *et al.*, 1998; Lametschwandtner *et al.*, 1998). The relatively high probabilities for the AKL-terminating proteins F08A8.2 and F08A8.3 to be mitochondrially located was due to amino acid sequences in their respective N termini that potentially represented mitochondrial leader sequences. Although not specified in the users' manual, it appears that in cases of dual targeting, the PSORT II algorithm allocates the protein preferentially to the mitochondria. In summary, the BLAST and PSORT II data in Table 4 combined with the two-hybrid results in Tables 3 and 5 were congruent with our hypothesis that nematodes might contain a peroxisomal acyl-CoA oxidase.

### Nematode proteins resembling yeast Fox2p

In *S. cerevisiae*, the second and third steps of classical  $\beta$ -oxidation are catalysed by Fox2p (Hiltunen *et al.*, 1992). From the outset, a computer search for Fox2p-like *C. elegans* proteins based on

Table 4. *C. elegans* proteins with similarities to *S. cerevisiae* classical  $\beta$ -oxidation enzymes

Yeast	yC <sup>a</sup>	Worm	Similarity	wC <sup>a</sup>	Subcellular location <sup>b</sup>			Similar to <sup>c</sup>
					pex	mit	cyt	
Pox1p	INK	C48B4.1	1.4E-54	SKL	78	11	11	Pox1p
		F58F9.7	1.3E-52	SKL	78	11	11	Pox1p
		F08A8.2	1.0E-49	AKL	17	35	21	Pox1p
		F59F4.1	1.3E-41	SKL	78	11	11	Pox1p
		F25C8.1	1.0E-36	AKM	4	9	52	Pox1p
		F08A8.3	8.2E-36	AKL	–	43	26	Pox1p
		F08A8.1	1.6E-34	SKL	78	11	11	Pox1p
		F08A8.4	9.1E-34	AKL	44	22	22	Pox1p
Fox2p	SKL	M03A8.1	5.9e-71	SKL	67	11	22	Fox2p
		E04F6.3	3.9e-44	SKL	78	11	11	Fox2p
		F09E10.3	3.2e-24	FSM	–	22	30	Fox2p
		Y39A1A.11	3.2e-18	LGM	9	4	65	Fox2p
		F54F3.4	6.4e-14	RFS	–	26	61	Sps19p,Fox2p
		D1054.8	5.8e-11	LLH	–	35	43	YMR226c,Sps19p
		T05F1.10	1.6e-10	FHK	4	22	22	YMR226c
		T02E1.5	1.9e-10	RQA	4	22	22	YDL114w
		C45B11.3	2.8e-10	SKL	78	11	11	Fox2p
		R05D8.b	3.8e-10	IDG	–	22	–	Sps19p
		F01G4.2	4.4e-10	MPA	4	22	40	YDL114w
		T11F9.11	7.4e-10	KDN	–	11	11	YDL114w,YMR226c
		R05D8.e	5.7e-09	SSV	–	30	26	YMR226c,Sps19p
		ZK829.1	1.3e-08	AKA	4	26	40	YMR226c,YIR035c
		F02C12.2	2.8e-07	GKK	4	40	26	YMR226c
C17G10.8	3.9e-07	GKL	4	22	39	Fox2p		
Pot1p	IKE	F53A2.7	4.1E-54	LGL	–	26	52	Erg10p,Pot1p
		T02G5.8	3.2E-47	QKL	–	74	22	Erg10p,Pot1p
		T02G5.7	4.2E-45	KKL	9	35	40	Erg10p,Pot1p
		B0303.3	9.4E-39	YGK	9	56	9	Pot1p,Erg10p
		T02G5.4	1.3E-22	QKL	4	52	22	Erg10p,Pot1p
		Y57A10C.6	0.048	SKI	9	13	52	Erg10p,Pot1p

<sup>a</sup>yC refers to C termini of yeast proteins, wC to those in worm orthologues.

<sup>b</sup>Numbers reflect the percentage probability predicted by the PSORT II algorithm for a particular protein to be localized either in peroxisomes (pex), mitochondria (mit) or the cytoplasm (cyt). Columns for the localization in other subcellular sites, such as the nucleus, endoplasmic reticulum, plasma membrane, Golgi, etc., are not shown.

<sup>c</sup>refers to yeast proteins most similar to the worm orthologue.

– no score available.

complete peptide sequences was likely to reveal a large subset of proteins belonging to the extended family of non-metallo short-chain alcohol dehydrogenases. In this and subsequent sections, only those proteins with singular characteristics were addressed experimentally. The protein most similar to Fox2p (M03A8.1; Table 4) is denoted by WormPD<sup>TM</sup> as FAT-2 to indicate that it is probably a fatty acid desaturase. However, since it ends with SKL (Table 4) and is also highly similar to the mammalian trifunctional enzyme (WormPD<sup>TM</sup>), it represents a good candidate for a peroxisomal  $\beta$ -oxidation protein. E04F6.3 (and to a

lesser extent C45B11.3) similarly appears to fulfil the latter two criteria (Table 4).

The protein under the Fox2p section with the least similar value (C17G10.8; Table 4) is the product of the *fat-3* gene. Proteins with a Fox2p similarity below that of FAT-3 were not listed. FAT-3 is an interesting protein because it terminates with a GKL tripeptide that interacts with human and yeast Pex5p (Lametschwandtner *et al.*, 1998), and potentially also with *C. elegans* PEX-5 (uppermost tripeptide, third column, Table 3). To determine whether FAT-3 could represent a peroxisomal protein, a Gal4p-GKL from the screen by

Table 5. Two-hybrid interactions between *C. elegans* PEX-5 and PTS1-extended protein partners

Strain	C terminus	$\beta$ -Galactosidase activity <sup>a</sup>	Relative level
yAG1257	M-HML <sup>b</sup>	216.4 $\pm$ 9.6	1.0
yAG1256	R-SKL	574.5 $\pm$ 23.2	2.7
yAG1262	W-AKL	631.4 $\pm$ 9.5	2.9
yAG1263	L-AKL	446.8 $\pm$ 22.2	2.1
yAG1264	F-AKL	352.0 $\pm$ 21.7	1.6
yAG1265	R-AKL	1430.3 $\pm$ 53.9	6.6
yAG1257	M-HML	177.0 $\pm$ 1.3	1.0
yAG1256	R-SKL	934.9 $\pm$ 89.0	5.3
yAG1266	A-AKM	293.4 $\pm$ 8.5	1.7
yAG1257	M-HML	177.0 $\pm$ 1.3	1.0
yAG1256	R-SKL	934.9 $\pm$ 89.0	5.3
yAG1267	Y-GKL	90.3 $\pm$ 2.4	0.5
yAG1269	R-GKL	143.0 $\pm$ 6.7	0.8
yAG1268	R-QKL	57.9 $\pm$ 4.6	0.3
yAG1257	M-HML	221.6 $\pm$ 5.5	1.0
yAG1256	R-SKL	774.6 $\pm$ 82.5	3.5
yAG1270	K-LKL	3.8 $\pm$ 0.3	0.0
yAG1271	D-LKL	4.4 $\pm$ 0.2	0.0

<sup>a</sup>nmol *O*-nitrophenyl- $\beta$ -D-galactoside hydrolysed/min/mg protein; mean  $\pm$  SD, *n* = 3.

<sup>b</sup>The amino acid to the left of the hyphen is adjacent to the C-terminal tripeptide and may be important for PTS1 function (Lametschwandner *et al.*, 1998).

Lametschwandner *et al.* (1998) was analysed for two-hybrid interaction with nematode PEX-5 (third cluster, Table 5). The results demonstrated that GKL gave rise to levels of  $\beta$ -galactosidase activities that were 0.5–0.8-fold of those obtained with HML (third cluster, Table 5). Therefore, the two-hybrid data for FAT-3 being a peroxisomal protein remain equivocal. FAT-2 (M03A8.1) and E04F6.3, on the other hand, might be promising as candidates for peroxisomal multifunctional enzymes.

#### Putative nematode thiolases

The last step of the  $\beta$ -oxidation spiral in *S. cerevisiae* is catalysed by Pot1p/Fox3p (Einerhand *et al.*, 1991; Igual *et al.*, 1991), whose import depends on Pex7p (Marzioch *et al.*, 1994). *C. elegans* has been previously reported to contain Y57A10C.6/P-44 (Bun-Ya *et al.*, 1997), a peroxisomal type-II 3-oxoacyl-CoA thiolase (Table 4). However, P-44 is similar to mammalian sterol carrier protein x (SCPx) and, therefore, does not represent a classical  $\beta$ -oxidation thiolase. Instead, it is thought to be

involved in phytanic acid degradation and bile acid synthesis (Bun-Ya *et al.*, 1997; Maebuchi *et al.*, 1999).

The C termini of the Pot1p/Fox3p-like potential  $\beta$ -oxidation thiolases identified here (Table 4) did not look like PTS1s, with the exception of QKL (extending from T02G5.4 and T02G5.8), which has previously been shown to interact with yeast Pex5p (Lametschwandner *et al.*, 1998) and possibly KKL (extending from T02G5.7). Our two-hybrid results showed that QKL was even less efficient than GKL as a nematode PEX-5 interaction partner, since it gave rise to  $\beta$ -galactosidase values that were only 0.3-fold of those generated using HML (third cluster, Table 5). An appropriate Gal4p fusion to determine whether KKL might be efficient at interacting with nematode PEX-5 was not available. This is because Gal4p–KKL had not turned up as a positive interaction partner, either in the present two-hybrid screen (Table 3) or in previous ones (Kragler *et al.*, 1998; Lametschwandner *et al.*, 1998). The possibility of KKL interacting with PEX-5 notwithstanding, the apparent absence of a potential nematode thiolase with an efficient PTS1 could mean that, like the situation in *S. cerevisiae* (and mammals), the putative *C. elegans* thiolase is also imported in a PEX-5-independent manner.

#### Auxiliary enzymes of $\beta$ -oxidation: potential nematode 2,4-reductases

From the data gathered on nematode Pox1p- and Fox2p orthologues, it is possible that *C. elegans* contains the components of a basic peroxisomal  $\beta$ -oxidation spiral for degrading saturated fatty acids. To break down unsaturated fatty acids with odd-numbered double bonds such as oleic acid (*cis*-C<sub>18:1(9)</sub>), yeast engage peroxisomal Eci1p (Gurvitz *et al.*, 1998; Geisbrecht *et al.*, 1998), corresponding to the auxiliary enzyme  $\Delta^3$ - $\Delta^2$ -enoyl-CoA isomerase. Eci1p is also critical for breaking down even-numbered *cis*-double bonds in fatty acids such as petroselinic acid (*cis*-C<sub>18:1(6)</sub>). Metabolism of petroselinic acid additionally requires Sps19p (Gurvitz *et al.*, 1997) that represents the auxiliary enzyme 2,4-dienoyl-CoA reductase (2,4-reductase). In mammals, 2,4-reductases are thought to also participate in degrading oleic acid (Smeland *et al.*, 1992).

A computer search for Sps19p orthologues revealed three well-scoring proteins, two of which (T05C12.3 and W01C9.4) were predicted to be



mitochondrial, and the one terminating with SKL (F53C11.3) to be peroxisomal (Table 6). Like the previous situation with Fox2p, a search for Sps19p-like nematode proteins was bound to reveal additional less-related dehydrogenases. For example, several of the worm proteins identified here are similar to other putative yeast dehydrogenases (Ymr226cp, Yir035cp, and Ydl114wp; right column, Table 6) which, however, do not represent 2,4-reductases, since the yeast *sps19Δ* mutant does not contain redundant isoenzymes allowing it to utilize petroselinic acid (Gurvitz *et al.*, 1997). The SKL tripeptide extending from F53C11.3 has been amply demonstrated in the present work to interact with *C. elegans* PEX-5 (Tables 3, 5). On the other hand, the tripeptides EKP and EKA extending from T05C12.3 and W01C9.4 (Table 6) do not conform to the PTS1 consensus and have not been isolated as Pex5p-interacting tripeptides in two-

hybrid screens (Lametschwandtner *et al.*, 1998). The lack of a canonical PTS1 in these two proteins was coincidental with a high probability for a mitochondrial location. The important implication of the putative distribution of the conjectured nematode 2,4-reductases to both peroxisomes and mitochondria is discussed in a later section.

### Nematode hydratase/isomerase proteins

Eci1p is 46% identical to Dci1p (Geisbrecht *et al.*, 1999a) which represents peroxisomal  $\Delta^{3,5}$ - $\Delta^{2,4}$ -dienoyl-CoA isomerase (Gurvitz *et al.*, 1999a). Dci1p contains a PTS1-like HKL C terminus but, like the HRL-terminating Eci1p, could be demonstrated to be imported into peroxisomes by an internal signal acting via a novel route (Karpichev and Small, 2000). Of the five potential nematode Eci1p or Dci1p orthologues shown (Table 6), only one

Table 6. *C. elegans* proteins with similarities to *S. cerevisiae*  $\beta$ -oxidation auxiliary enzymes

Yeast	yC	Worm	Similarity	wC	Subcellular location			Similar to
					pex	mit	cyt	
Sps19p	SKL	T05C12.3	1.2e-29	EKP	4	48	30	Sps19p
		W01C9.4	8.8e-29	EKA	–	65	13	Sps19p
		F53C11.3	3.8e-28	SKL	78	11	11	Sps19p
		R05D8.b	8.7e-22	IDG	–	22	–	Sps19p
		R05D8.d	1.8e-21	TAR	4	9	13	Sps19p
		D1054.8	1.6e-20	LLH	–	35	43	YMR226c,Sps19p
		T01G6.1	1.6e-20	LTG	–	56	35	YMR226c,Sps19p
		F28H7.2	4.9e-19	KEL	–	39	43	YMR226c
		R05D8.c	6.3e-19	SQE	–	35	–	YMR226c,Sps19p
		C01G12.5	1.0e-18	LKH	–	65	22	YMR226c,Sps19p
		F25D1.5	1.3e-18	LSQ	–	35	48	YMR226c,Sps19p
		F54F3.4	5.7e-18	RFS	–	26	61	Sps19p,Fox2p
		C06E4.3	7.2e-18	PQK	4	–	35	YMR226c,Sps19p
		F26D2.15	1.2e-16	LKA	4	35	35	YMR226c,Sps19p
		R05D8.e	2.3e-16	SSV	–	30	26	YMR226c,Sps19p
		F09E10.3	7.2e-16	FSM	–	26	48	Fox2p
		R08H2.1	1.4e-15	LNQ	4	35	35	YMR226c
		ZK829.1	4.1e-14	AKA	4	26	48	YMR226c,YIR035c
		Y39A1A.11	5.6e-14	LGM	4	–	61	Fox2p
		F02C12.2	2.2e-13	GKK	4	43	26	YMR226c
C06E4.6	2.7e-13	PRQ	4	17	43	YMR226c		
W03F9.9	1.4e-12	RAE	–	9	65	YMR226c		
F01G4.2	6.5e-12	MPA	4	4	52	YDL114w		
M03A8.1	3.3e-11	SKL	56	22	22	Fox2p		
Eci1p	HRL	R06F6.9	1.6E-20	AKK	–	44	26	E/Dci1p,Acblp
Dci1p	HKL	T05G5.6	9.3E-10	ESK	–	52	30	YDR036c,Eci1p
		F58A6.1	4.5E-07	SKL	78	11	11	YDR036c,Eci1p
		B0272.4	7.9E-07	SKI	–	11	11	YDR036c,E/Dci1p
		F38H4.8	7.4E-05	KKN	–	91	4	YDR036c,E/Dci1p

contained an obvious PTS1 (F58A6.1), whereas the remaining proteins were predicted as being predominantly mitochondrial. Although SKI acts as PTS1 for *Hansenula polymorpha* catalase (Didion and Roggenkamp, 1992), SKI and AKK are not considered as potential PTS1s in *S. cerevisiae* or humans (Lametschwandtner *et al.*, 1998).

Dcilp and Eci1p belong to the low similarity hydratase/isomerase family of proteins, all of which act on CoA-ester substrates and some are additionally entrained in  $\beta$ -oxidation (Müller-Newen *et al.*, 1995). Other members of this family include rat mitochondrial/peroxisomal ECH1 (Filppula *et al.*, 1998) and peroxisomal MFE type 1 (Palosaari and Hiltunen, 1990), naphthoate synthase, 4-chlorobenzoyl-CoA dehalogenase, carnitine racemase, 3-hydroxyisobutyryl-CoA hydrolase (Müller-Newen *et al.*, 1995), and *S. cerevisiae* Ydr036cp (Gurvitz *et al.*, 1999a). An additional computer search for further hydratase/isomerase proteins based on similarity to Ydr036cp (Table 7) revealed at least two nematode proteins that might represent peroxisomal  $\Delta^3$ - $\Delta^2$ -enoyl-CoA isomerases: F09F7.4 ending with LKL, and the SKL-terminating protein F43H9.1. However, these data are not sufficient to substantiate our theory that nematodes might contain the requisite process for degrading oleic acid.

*C. elegans* F09F7.4 and *S. cerevisiae* Ydr036cp, found when searching for Eci1p and Dcilp orthologues, are the two most similar proteins in the respective species (45 and 33%, respectively) to human 3-hydroxyisobutyryl-CoA hydrolase (Hawes *et al.*, 1996). A deficiency in this enzyme is lethal to humans (Brown *et al.*, 1982), and a decreased level of this activity is thought to be a cause of liver failure in cirrhosis (Taniguchi *et al.*, 1996). Like the

human enzyme, which is located in the mitochondria, the putative nematode protein was predicted by the algorithm to be similarly mitochondrial. However, this novel protein additionally terminates with LKL which, at least in *S. cerevisiae*, represents a Pex5p-interacting PTS1 (Lametschwandtner *et al.*, 1998). To determine whether LKL could represent a functional PTS1 in *C. elegans*, it was assayed for two-hybrid interaction (Table 5). The results showed that LKL was significantly less efficient compared to SKL or even HML in interacting with nematode PEX-5, yielding  $\beta$ -galactosidase activities that were essentially below the detection level of the system used (bottom panel, Table 5). We therefore reaffirm the algorithm's prediction that F09F7.4 is an extra-peroxisomal protein.

## Discussion

Combining experimental data with results from computer searches offers a powerful method for making meaningful predictions regarding the function of novel proteins. Here, we wanted to know whether *C. elegans* contains the full set of peroxisomal  $\beta$ -oxidation enzymes analogous to that found in *S. cerevisiae*, including those enzymes essential for degrading unsaturated fatty acids. For this purpose, we have identified nematode PEX-5. This meant that *C. elegans* contains at least one component of the peroxisomal import machinery known to exist in other organisms, including yeast, plants and mammals. Such homology studies using yeast *PEX* genes have been instrumental in identifying the corresponding human genes (Subramani, 1997). By following a similar approach, homology probing should allow the isolation of all the potential *C. elegans* peroxin genes (i.e. involved in peroxisome biogenesis). We have also identified potential orthologues of yeast proteins involved in  $\beta$ -oxidation. Since several of these orthologues terminated with a PTS1 that could interact with nematode PEX-5 in the yeast two-hybrid assay, we predict that at least some of them represent peroxisomal  $\beta$ -oxidation proteins.

Regarding those orthologues not containing an efficient PEX-5-interacting C terminus, such as LKL or GKL (F09F7.4 and FAT-3, respectively), it should be noted that in certain yeast peroxisomal proteins PTS1 occurs in a redundant system with additional cryptic PTSs. Examples for this are given

Table 7. YDR036c-like *C. elegans* proteins with a hydratase/isomerase fingerprint

Worm	Similarity	wC	Subcellular location			Similar to
			pex	mit	cyt	
F09F7.4	7.5e-39	LKL	4	48	35	YDR036c
F56B3.5	1.8e-14	KRD	9	44	39	YDR036c
C29F3.1	9.5e-11	FYS	–	100	–	YDR036c
T08B2.7	5.4e-10	FYN	–	22	48	YDR036c
F43H9.1	5.1e-09	SKL	67	11	22	YDR036c
Y25C1A.a	4.9e-06	EDV	–	65	17	YDR036c, E/Dcilp

by Cta1p (Kragler *et al.*, 1993), Cat2p (Elgersma *et al.*, 1995), Eci1p (Karpichev and Small, 2000) and Dci1p (Karpichev and Small, 2000). Therefore, although we do not provide positive evidence for this, we hypothesize that in such cases of type 1 signals interacting only weakly with nematode PEX-5, these could be augmented by internal signals. It is also not clear whether nematodes actually have PTS2-containing proteins or the cognate receptor, PEX-7. A clue to the existence of PTS2 proteins could have come from the identification of PTS1-less proteins with a high probability for peroxisomal localization. However, inspection of Tables 4 and 6 does not reveal any PTS2 proteins (including the putative thiolases), since all those orthologues to which were attributed high probabilities for a peroxisomal location were actually terminated with a canonical PTS1. In addition, a search of WormPD<sup>TM</sup> for a potential PEX-7 revealed a very large protein, Y32H12A.8 (and its putative paralogue Y32H12A\_57.A), consisting of 3885 amino acids with only a weak similarity (26% identity over 246 amino acids) to the 323 amino acid-long human PEX7. Therefore, the issue of the existence of a PEX-7-dependent peroxisomal import mechanism remains open for the time being.

As proposed in the Introduction, were (the putative peroxisomal)  $\beta$ -oxidation in *C. elegans* to be augmented by a mitochondrial process, such as occurs in mammals but not yeast or plants (Kunau *et al.*, 1995), nematodes could prove to be a better model than *S. cerevisiae* for studying the relationship between the two compartmentalized processes. A principal difference between the two processes is that the first step in peroxisomal  $\beta$ -oxidation is initiated by an acyl-CoA oxidase, whereas that in mitochondria is executed by an acyl-CoA dehydrogenase. A search for proteins with similarities to acyl-CoA dehydrogenase revealed 13 potential mitochondrial proteins (WormPD<sup>TM</sup>). The PSORT II results presented here, predicting that at least some of the yeast-like  $\beta$ -oxidation orthologues contained mitochondrial leader signals, is also a fairly good indication that nematodes might resemble mammals in having an additional mitochondrial  $\beta$ -oxidation compartment. This could extend the nematode's potential versatility for studying human disorders associated with deficient mitochondrial  $\beta$ -oxidation.

For example, human 2,4-dienoyl-CoA reductase

deficiency (OMIM number 222745; 24 February 2000) is thought to be due to a defective mitochondrial 2,4-reductase (Roe *et al.*, 1990). A candidate human mitochondrial enzyme has been identified (Koivuranta *et al.*, 1994) and shown to be a physiological 2,4-reductase devoid of any cryptic PTSs (Gurvitz *et al.*, 1999b). Although a yeast 2,4-reductase knockout has been generated (Gurvitz *et al.*, 1997), this mutant fails to serve as an appropriate model for studying the underlying mechanism behind this deficiency, for two reasons. First, the missing yeast enzyme is peroxisomal, and second, unlike the human patient, which could not degrade odd-numbered *cis*-double bonds properly (Roe *et al.*, 1990), the mutant yeast strain is not affected for this function (Gurvitz *et al.*, 1997).

The distribution of the putative 2,4-reductases in the nematode is reminiscent of that in mammals (Table 6); rodents and probably also humans contain one peroxisomal 2,4-reductase (Fransen *et al.*, 1999; Geisbrecht *et al.*, 1999b) and two mitochondrial isoenzymes (Hakkola *et al.*, 1989; Hakkola and Hiltunen, 1993). Hence, in light of the limitations of using yeast cells which have only peroxisomal  $\beta$ -oxidation, *C. elegans* might prove to be a more appropriate test organism for examining the effect of knocking out the mitochondrial 2,4-reductases on growth on oleic acid. Although several mouse knockouts for  $\beta$ -oxidation enzymes exist, the effect of removing the respective proteins in worms might be more easily studied.

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