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RNA interference of peroxisome-related genes in *C. elegans*: a new model for human peroxisomal disorders

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Petriv, Oleh I., David B. Pilgrim, Richard A. Rachubinski, and Vladimir I. Titorenko. RNA interference of peroxisome-related genes in *C. elegans*: a new model for human peroxisomal disorders. *Physiol Genomics* 10: 79–91, 2002. First published July 2, 2002; 10.1152/physiolgenomics.00044.2002.—RNA-mediated interference (RNAi) for the posttranscriptional silencing of genes was used to evaluate the importance of various peroxisomal enzymes and peroxins for the development of *Caenorhabditis elegans* and to compare the roles of these proteins in the nematode to their roles in yeasts and humans. The nematode counterparts of the human ATP-binding cassette half-transporters, the enzymes alkyldihydroxyacetonephosphate synthase and $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase, the receptors for peroxisomal membrane and matrix proteins (Pex19p and Pex5p), and components of the docking and translocation machineries for matrix proteins (Pex13p and Pex12p) are essential for the development of *C. elegans*. Unexpectedly, RNAi silencing of the acyl-CoA synthetase-mediated activation of fatty acids, the α- and β-oxidation of fatty acids, the intraperoxisomal decomposition of hydrogen peroxide, and the peroxins Pex1p, Pex2p, and Pex6p had no apparent effect on *C. elegans* development. The described analysis of functional gene knockouts through RNAi provides a basis for the use of *C. elegans* as a valuable model system with which to study the molecular and physiological defects underlying the human peroxisomal disorders.

RNA-mediated interference; peroxin; cyan fluorescent protein; protein targeting; Zellweger syndrome

THE GENOME OF THE NEMATODE, *Caenorhabditis elegans*, one of the first genomes of a multicellular organism to be sequenced, contains nearly 19,000 potential genes (58). Although 40% of these genes have apparent homologs in the human genome (52), their functions for the most part remain unknown. The posttranscriptional silencing of genes by RNA-mediated interference (RNAi) provides a convenient methodology with which to examine gene function in *C. elegans* (25). Some features of the nematode, such as a complex nervous system, short reproduction time, well-established life cycle, and transparent cuticle make this organism particularly suited to the study of peroxisome biogenesis, function, and pathology, compared with classic model organisms such as yeasts or mice. In addition, some features of peroxisome biogenesis in *C. elegans*, such as the lack of a machinery for the import of proteins sorted to this organelle by the peroxisomal targeting signal-2 (PTS2) pathway as demonstrated by Motley and coworkers (39), clearly distinguish this organism from yeast and mouse model systems (39, 65).

Peroxisomes are organelles found in most eukaryotic cells (46, 55, 64). They show remarkable morphological and metabolic plasticity, as their number, size, morphology, protein composition, and biochemical functions vary depending on the organism, cell type, and/or environmental milieu (40, 51, 63, 66). Typically, peroxisomes contain at least one oxidase that produces harmful hydrogen peroxide and catalase, a hydrogen peroxide-degrading enzyme (6). In human and other mammalian cells, peroxisomes compartmentalize a variety of degradative and biosynthetic metabolic pathways. Degradative pathways include the α- and β-oxidation of fatty acids, the catabolism of purines and amino acids, and the breakdown of prostaglandins and polyamines (52, 71). Biosynthetic pathways include the formation of plasmalogens, cholesterol, bile acids, and polyunsaturated fatty acids (30, 52, 70).

Peroxisomes are indispensable for normal human development and physiology, as shown by the severity and lethality of numerous peroxisomal disorders (17, 54, 55, 70). These disorders can be divided into two groups, the peroxisome biogenesis disorders (PBDs) and the peroxisomal single enzyme disorders (9, 13, 17, 42, 68–71). The PBDs affect multiple peroxisomal metabolic pathways, as patients fail to assemble functionally intact peroxisomes (13, 17, 46, 55). A hallmark of the Zellweger spectrum of PBDs, including Zellweger syndrome (ZS) itself, neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD), is the global developmental delay caused by the incomplete migration of neuroblasts during development (38, 41, 68). Patients with ZS rarely survive their first year, whereas NALD and, especially, IRD are less severe disorders, with some patients surviving into their third and fourth decade (17, 68). Another distinct form of PBD, rhizomelic chondrodysplasia punctata (RCDP)

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type 1, is characterized clinically by abnormal psychomotor development, mental retardation, and death in early infancy (43). The PBDs are inherited in an autosomal recessive fashion, with an incidence of ~1 in 50,000 live births (46).

The second group of disorders, peroxisomal single enzyme disorders, results from mutations in genes encoding single peroxisomal enzymes (45, 68, 70, 71). The most common of these disorders, X-linked adrenoleukodystrophy (X-ALD) (71), occurs in ~1 in 15,000 live births (46). X-ALD is caused by defects in ALDP, an integral peroxisomal membrane protein (PMP) that is a member of the ATP-binding cassette (ABC) half-transporter family of proteins (10). Single defects in many other peroxisomal enzymes involved in the α- and β-oxidation of fatty acids, amino acid catabolism, and the biosynthesis of plasmalogens and bile acids have also been shown to cause peroxisomal disorders (68, 70, 71).

Peroxisome assembly, division, and inheritance are regulated by at least 23 proteins called peroxins that are encoded by the *PEX* genes, and mutations in 11 of these genes cause the PBDs (13, 17, 55). Peroxisomal proteins are encoded by nuclear genes, synthesized on cytosolic polysomes and imported posttranslationally across the peroxisomal membrane (46, 55, 64). They are sorted to the organelle by specific PTSs. These PTSs are recognized in the cytosol by their cognate receptors, which guide the targeting and docking of proteins to the peroxisomal membrane (21, 57). Most matrix proteins are targeted to the peroxisome by PTS1, a carboxy-terminal tripeptide with the consensus motif (Ser/Ala/Cys)(Lys/Arg/His)(Leu/Met/Ile), and its cytosolic shuttling receptor Pex5p (20, 21, 46, 52, 64). A few matrix proteins are targeted by a distinct PTS2, an amino-terminal nonapeptide with the consensus motif (Arg/Lys)(Leu/Val/Ile)Xaa_5(His/Gln)(Leu/Ala), and its cytosolic shuttling receptor Pex7p (21, 55). As reported above, the PTS2 pathway is absent in *C. elegans* (39). The initial docking site for both the Pex5p-PTS1 and Pex7p-PTS2 complexes on the cytosolic surface of the peroxisomal membrane consists of three membrane-associated peroxins, Pex13p, Pex14p, and Pex17p (46, 64). After docking, the PTS1 and PTS2 receptors, together with their cargoes, are transferred to other membrane-associated components of the import machinery, including Pex2p, Pex10p, and Pex12p (21, 57). These components function in the translocation of cargo proteins, either alone or together with their receptors, across the peroxisomal membrane into the peroxisomal matrix (13, 55). In human cells, Pex2p, together with two cytosolic peroxins, Pex1p and Pex6p, may act to recycle the PTS1 receptor to the cytosol (13, 17, 46). Many PMPs are sorted to peroxisomes directly from the cytosol by a distinct membrane PTS, called mPTS1, and its putative cytosolic receptor, Pex19p (47, 57), which may also act as a chaperone to assist the assembly of PMPs in the peroxisomal membrane (55, 64).

The present study not only enhances our understanding of the functioning of the *C. elegans* peroxisomal protein import machinery but also sheds new light on the recently discovered role for peroxisomes in the process of development. Peroxisomes do more than just burn fat and dump toxic waste; they appear to be dynamically integrated into specific cell and organismal developmental programs, with their biogenesis being tightly coordinated with the biogenesis of other cellular compartments (1, 28, 32, 36, 49, 50, 57, 60–62). Here, we report the use of RNAi for the creation of an extensive collection of nematode functional gene knockout mutants deficient in a wide range of peroxisome-related functions. The availability of such a collection is a prerequisite for the use of *C. elegans* as an advantageous model system with which to study the molecular and physiological defects underlying the human peroxisomal disorders. We confirm the findings of Motley and coworkers (39) of the requirement for the enzyme alkyldihydroxyacetonephosphate synthase (ADHAPS) for *C. elegans* development, and, in addition, we define subsets of other peroxisomal enzymes and of peroxins essential for this program.

MATERIALS AND METHODS

Strains, Plasmids, and Germ Line Transformation

Wild-type worms (*C. elegans* variety Bristol, strain N2) were grown as described (31). DP9 unc-119 (e2498) was used as a host strain for the genomic integration of an extrachromosomal array encoding cyan fluorescent protein (CFP) tagged at its carboxyl terminus with the PTS1 Ser-Lys-Leu (CFP-SKL). The plasmid pPD118.25 (a gift of Dr. A. Fire) carried the CFP-SKL construct under the *let-858* promoter. The plasmid pDP#MM016 containing the *unc-119* cDNA was used as a dominant coinjection marker (33). Germ line transformation was carried out as described (35).

dsRNA Interference

The template for double-stranded RNA (dsRNA) synthesis was generated by PCR of *C. elegans* genomic DNA with oligonucleotide flankers encoding the T7 promoter and a specific sequence complementary to the gene of interest (Fig. 1). RNA was produced using the MEGAscript kit for RNA synthesis (Ambion, Austin, TX). After synthesis, both strands of RNA were annealed by incubation at 65°C for 10 min followed by slow cooling. dsRNA at a concentration of 0.5–1.0 mg/ml in 2% PEG-6000, 20 mM potassium phosphate, 3 mM potassium citrate, pH 7.5, was injected into the gonads of one or both gonad arms of young adult hermaphrodites (20–30 animals), as described (35). Injected animals were transferred to nematode growth medium (NGM) agar plates (4) containing the OP50 strain of *Escherichia coli*. After 12 h, the surviving animals were transferred to fresh culture plates, on which they were allowed to lay eggs. The percentage of adult F1 progeny was evaluated 3 days after injection. In RNAi experiments, the percentage of adults in the offspring of worms injected with control dsRNA was set at 100% in each independent experiment.

Genomic Integration of the CFP-SKL Expression Construct

Strain DP9 was injected with the plasmid pPD118.25 containing the CFP-SKL construct downstream of the *let-858* promoter. Plasmid pDP#MM016 containing the whole *unc-119* gene, including its promoter and 3′-untranslated region,

ORF name	ORF similarity to H. sapiens proteins	Oligonucleotides sequence 5'->3' not showing T7 promoter sequence Gene and dsRNA structure.
C11H1.6	**PEX1** 72% (2e-61)	AGCTCGTTCATGTGCCCCG TTAGGCTAACGTCACTTTCTGACCTATC
ZK809.7	**PXMP3** 39% (1e-10)	ATGATTCACCACTTTAGGTACCACCA TTGAGCAGACTCTTCTAGAAAATAGAAAGATAC
C34C6.6	**PXR1** 49% (6e-66)	AAGCAAACCCTTTCACAACCATG CTAGACTAGAGAGGCTTTTACAGCTGCC
F39G3.7	**PEX6** 56% (6e-67)	CTCAGATTTTCAAAAACTAATATTTAGTTTCATG AATAATCCTATAAACCACAATTTATTTCTCAGA
F08B12.2	**PEX12** 56% (7e-50)	GAGCGTCGTTCACGGAAAACT TTGTTATAAGTGTTTACGTATTGGTTGATACA
F32A5.6	**PEX13** 56% (2e-42)	GTATATTGTTTAGGTATGGTGGTGGGG TGAATTCAAATCTCTTGCTGGAAAAG
F54F2.8	**PXF** 45% (2e-19)	ATGACTGACGAAACAACCCAAAATAT CTCGAATTGTTTCTCATATCGTTCCT
Y54G11A.5B	**CAT** 80% (0.0)	ATGCCAAACGATCCATCGG ACCTCAGCGAAGTAGTTCCTTGG
T02G5.4 **T02G5.7** **T02G5.8**	**ACAT1** 59% (5e-74) **ACAT1** 69% (e-102) **ACAT1** 72% (e-120)	TTG(T/G)(T/C)G(G/T)(A/T)GGAA(T/C)(G/T)GA(G/A)A(G/A)(T/C)ATG(T/A)(G/C)(A/T)CAAGT(A/T)CCATTTT TCC(G/A)T(C/T)(G/A)CAAA(G/T)(G/T)GCAGC(T/A)AC(T/A)CCGA
Y57A10C.6	**SCP2** 82% (1e-59)	AACCTGCTGTGGTCAACGTGC TCAAATCTTGGACTGTGCAGCTC
Y50D7A.7	**AGPS** 71% (0.0)	ATGTCGGCGTCCTATCAAACAA TCTACAACTTGCAATGCGGTGA
Y65B4BL.5	**FACL1,2,5** 67% (0.0) to 70% (e-180)	GATGGTTCCCGCATCAGC CTCAGCATCAGGTACAACAATAGCA
F28D1.9	**VLCS-H1** 57% (e-101) **FATP4** 58% (e-127)	CAGGGTCTTGGCTATCGATCC TCAACATCCTGACAAATTCGCA
T02D1.5 **C44B7.9** **C44B7.8**	**ABCD2,3** 55% to 78% (0.0)	(G/C)(A/T)GAAGAAAT(T/C)GCATT(T/C)TA(C/T)(G/C)(G/A)AGG CCTTCAACATC(T/A)A(T/C)(T/A)(G/C)(T/A)(T/A)ACTGCAGA(G/A)GT
Y25C1A.13	**ECH1** 63% (3e-66)	ATGCTCGCCTCTCGCCGT TCTAATCGAGAAAATCGCATCCTGT

Fig. 1. Open reading frames (ORFs) targeted by RNA-mediated interference (RNAi). The similarity to human homologs (percentage and E value) is shown. The sequences of oligonucleotides used to generate double-stranded RNA (dsRNA) for individual ORFs are presented. The high level of homology between ORFs T02G5.4, T02G5.7, and T02G5.8, as well as between ORFs T02D1.5, C44B7.8, and C44B7.9, permitted the use of redundant oligonucleotides. The sequence of the T7 promoter region found at the 5′ end of every primer was 5′-TAATAC-GACTCACTATAGGGAGA.

was used as a positive marker in coinjections. Worms that reverted from the unc$^-$ phenotype to the wild-type phenotype were selected as containing extrachromosomal arrays. These worms were exposed to an ultraviolet radiation dose of 350 μJ using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Homozygotes stably expressing the CFP-SKL construct were selected.

Preparation of Anti-Thiolase Antibodies and Immunoblot Analysis

The last exon of the gene Y57A10C.6 encoding the 242 carboxyl-terminal amino acids of the protein P-44 was fused in-frame and downstream of the open reading frame (ORF) encoding *E. coli* maltose binding protein (MBP) in the vector pMAL-p2X (New England BioLabs, Beverly, MA). The recombinant protein product was purified by affinity chromatography on amylose resin and cleaved with factor Xa. The thiolase peptide was separated from MBP by SDS-PAGE, excised from the gel, and isolated by electroelution. Antibodies to the peptide were raised in rabbit and guinea pig. Immunoblot analysis and detection of antigen-antibody complexes by enhanced chemiluminescence were performed as described (60).

Immunofluorescence Microscopy

Double-labeling, immunofluorescence microscopy was performed as described (37). Rabbit and guinea pig anti-thiolase antibodies were used at a dilution of 1:100. Affinity-purified rabbit antibodies to green fluorescent protein (GFP) (a gift from Dr. L. Berthiaume, Department of Cell Biology, University of Alberta) were used at a dilution of 1:500. Primary antibodies were detected with rhodamine- or FITC-conjugated donkey anti-rabbit IgG antibodies or with rhodamine- or FITC-conjugated donkey anti-guinea pig IgG antibodies (Jackson, West Grove, PA). Samples were viewed with an Olympus BX50 microscope.

RESULTS

In Silico Search for C. elegans Orthologs of Human Peroxisomal Enzymes and Peroxins

To identify potential orthologs of human peroxisomal enzymes involved in lipid metabolism, and of peroxins, we performed a computer search of the *C. elegans* genome WormPD (**http://www.incyte.com/sequence/proteome/index.shtml**) and the Sanger Centre (**http://www.sanger.ac.uk/**) databases using the Gapped BLAST (v2.0.10) (**http://www.ncbi.nlm.nih.gov/blast/**) algorithm with SEG and COIL filters. Alignments were refined by the Smith-Waterman alignment algorithms from the UCS Sequence alignment package (v 2.0). A comprehensive list of the orthologs chosen for posttranscriptional silencing of their encoding genes by RNAi, along with a graphical representation of the genes themselves from WormBase (**http://www.wormbase.org/**) is presented in Fig. 1.

The following candidate *C. elegans* orthologs of human peroxisomal enzymes implicated in lipid metabolism and peroxins were identified.

Orthologs of human thiolases. Two human peroxisomal thiolases have been identified. Peroxisomal thiolase 1 (pTH1) acts only on straight-chain substrates and is not reactive with 3-ketoacyl-CoA esters carrying a methyl group at the α-carbon (20, 71). pTH1 is therefore not involved in the α-oxidation of very-long-chain fatty acids (VLCFAs) (20, 70). In silico searching revealed three candidate *C. elegans* orthologs of human pTH1: T02G5.8, T02G5.7, and T02G5.4. The carboxy termini of all three potential *C. elegans* orthologs of human pTH1 carry tripeptides that closely resemble the PTS1 signal for matrix proteins, namely QKL for T02G5.8, KKL for T02G5.7, and QKL for T02G5.4, all of which have been shown to function in targeting proteins to peroxisomes in other organisms (39). Previous studies have also predicted that *C. elegans* ORFs T02G5.8, T02G5.7, and T02G5.4 represent orthologs of human pTH1 (18, 39).

Peroxisomal thiolase 2 (pTH2) is the second human peroxisomal thiolase. pTH2, which is alternatively termed SCP2/3-ketothiolase or SCPx (for "sterol carrier protein x"), is required for the metabolism of 3-oxoacyl-CoA esters with a methyl branched chain at the α-carbon (20, 71). These CoA esters include intermediates of peroxisomal α-oxidation of 3-methyl branched VLCFAs, such as cerebronic and phytanic acids. The α-oxidation of VLCFAs has been shown to be impaired in ZS patients (27, 48). During α-oxidation, phytanic acid is shortened to pristanic acid, which then enters peroxisomal β-oxidation, with the last step catalyzed by pTH2 (20, 71). In silico searching revealed the ORF Y57A10C.6 of *C. elegans* as a potential ortholog of human pTH2. This ORF encodes the *C. elegans* peroxisomal thiolase, P-44 (34), which is required for the metabolism of 3-oxoacyl-CoA esters with a methyl branched chain at the α-carbon (65).

Orthologs of human catalase. Catalase converts hydrogen peroxide to water and molecular oxygen. In silico searching revealed two candidate *C. elegans* orthologs, Y54G11A.6 and Y54G11A.5b, of the only known human catalase. These *C. elegans* ORFs have been shown to encode two distinct forms of catalase, a cytosolic catalase, ctl-1 (56), and a peroxisomal catalase, ctl-2 (65). Notably, mutations in the *ctl-1* gene reduce the adult lifespan (56). Peroxisomal catalase ctl-2 encoded by ORF Y54G11A.5b contains a PTS1 tripeptide at its carboxyl terminus.

Ortholog of human ADHAPS. ADHAPS catalyzes the second step in the biosynthesis of plasmalogen in peroxisomes (19). In humans, defects in ADHAPS result in a peroxisomal single enzyme disorder, RCDP type 3 (9). ADHAPS, which is targeted to peroxisomes by a PTS2 in mammals (7), contains a PTS1 in *C. elegans* (8). The *C. elegans* counterpart of human ADHAPS is encoded by ORF Y50D7A.7 (39).

Orthologs of human ABC half-transporters. Four human peroxisomal membrane ABC half-transporters have been identified, namely ALDP, ALDRP, PMP70, and PMP69 (70, 71). They are all members of the so-called ABCD subfamily of the ABC transporter superfamily (10). Different combinations of ABC half-transporters to form dimers (peroxisomal half-transporter heterodimers) have been proposed to mediate the transport of distinct subsets of their substrates across the peroxisomal membrane (10). ALDP is impli-

cated in the uptake of VLCFA-CoAs, as a deficiency in this ABC half-transporter leads to the accumulation of $C_{26:0}$ VLCFA-CoA in the plasma of patients suffering from X-ALD (23). The functions of the other three transporters remain to be established, although, considering their high similarity to each other and to ALDP, they may be involved in fatty acid transport across the peroxisomal membrane (10). Indeed, PMP70 has recently been implicated in the peroxisomal transport of 2-methylacyl-CoA esters (71). In silico searching revealed three candidate *C. elegans* orthologs of human ALDP, namely, T02D1.5, C44B7.9, and C44B7.8. The proteins encoded by these *C. elegans* ORFs also show high similarity to two human ALDP-like transporters, ABCD2 and ABCD3. T02D1.5 is also a putative ortholog of human ALDRP. Of note, the ORFs C44B7.8 and C44B7.9, as well as the ORFs T02G5.7 and T02G5.8, are linked, being separated by ~380 bp and 220 bp, respectively. Therefore, their levels of expression may be modulated by common regulatory elements.

Orthologs of human acyl-CoA synthetases. An initial step of fatty acid metabolism in humans is catalyzed by several acyl-CoA synthetases, which all ligate CoA to free fatty acid but differ in their substrate specificities from VLCFAs to long-chain fatty acids (LCFAs) (14, 41, 51). Acyl-CoAs formed in the acyl-CoA synthetase-catalyzed reaction are key intermediates in the biosynthesis of various lipids, including triacylglycerols, phospholipids, cholesterol esters, and sphingomyelin, and in the β-oxidation of fatty acids (70, 71). *C. elegans* fatty acid transport protein a (*Ce*FATPa) is a candidate ortholog of two human acyl-CoA synthetases, heart-specific very-long-chain fatty acyl-CoA synthetase-related protein (VLCS-H1) and fatty acid transport protein 4 (FATP4) (22), and is encoded by ORF F28D1.9. Oversynthesis of *Ce*FATPa in COS cells greatly increases their LCFA uptake (22).

The protein encoded by *C. elegans* ORF Y65B4BL.5 is an ortholog of two *Saccharomyces cerevisiae* peroxisomal acyl-CoA synthetases, Faa1p and Faa2p, and shows similarity to three human acyl-CoA synthetases, FACL1, FACL2, and FACL5.

Ortholog of human $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase. This enzyme catalyzes the first step of auxiliary reactions that allow polyunsaturated fatty acids to enter the normal pathway for the β-oxidation of fatty acids (12). A candidate *C. elegans* ortholog of human peroxisomal $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase-like protein ECH1 is encoded by ORF Y25C1A.13.

Orthologs of human peroxins. Several functional categories of human peroxins have been chosen for the *in silico* search of their potential *C. elegans* counterparts. A candidate ortholog of the human PTS1 receptor, Pex5p, is encoded by ORF C34C6.6. An analysis of the interactions between the *C. elegans* ortholog of human Pex5p and PTS1-containing peroxisomal matrix proteins revealed that the Pex5p-dependent route for the peroxisomal targeting of PTS1 proteins is conserved between humans and nematodes (18). A potential ortholog of human peroxin Pex19p is encoded by ORF F54F2.8. Candidate orthologs of Pex13p and Pex12p are encoded by ORFs F32A5.6 and F08B12.2, respectively, while potential orthologs of human Pex2p, Pex1p, and Pex6p are encoded by ORFs ZK809.7, C11H1.6, and F39G3.7, respectively (15).

Peroxisomal Enzymes and Peroxins Implicated in the Development of C. elegans

We used RNAi for the epigenetic inactivation of genes encoding *C. elegans* orthologs of human peroxisomal enzymes and peroxins, and the effects of post-transcriptional silencing of the individual genes on normal development of the nematode were evaluated. ORFs were targeted individually in the RNAi experiments, with the exception of those encoding *1*) the three ABC half-transporters, *2*) the three orthologs of human pTH1, *3*) the three orthologs of human pTH1 and the ortholog of human pTH2, and *4*) the orthologs of human peroxins Pex1p and Pex6p. These four groups of orthologous proteins were targeted by coinjection of dsRNAs specific for the individual genes coding for the proteins of a particular group.

The offspring of worms injected with dsRNAs specific to ORFs for various peroxisomal enzymes of lipid metabolism can be divided into two distinct phenotypes (Fig. 2). The first phenotype showed significant developmental delay in the worms, with almost no adult worms being found 3 days postinjection. The second phenotype was almost indistinguishable from that of control worms, and, compared with control values, 60–100% of the F1 progeny of worms injected with dsRNA reached adult stage.

No adults were found in the offspring of worms 3 days after injection with dsRNAs specific to the genes for ADHAPS, $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase, or the three ABC half-transporters (Fig. 2). Most of these worms were the size of larvae at the L1, L2, or L3 stage. In contrast, at the same point of time, large numbers of adults and eggs were observed in the offspring of worms injected with either control dsRNA or dsRNAs specific to genes encoding type 2 thiolase P-44, the three type 1 thiolases, the three type 1 thiolases and thiolase P-44 (data not shown), catalase ctl-2, the ortholog of yeast acyl-CoA synthetases Faa1p and Faa2p encoded by ORF Y65B4BL.5, or the ortholog of human VLCFA-CoA synthetase encoded by ORF F28D1.9 (Fig. 2). Nevertheless, injection of dsRNA to the gene encoding type 2 thiolase P-44 was effective in reducing the amount of this protein to ~10% of its normal levels (Fig. 3), demonstrating the effectiveness of RNAi in the posttranscriptional silencing of specific genes.

We then evaluated the effect of RNAi on *C. elegans* genes encoding various peroxins. The offspring of worms 3 days following injection with dsRNAs specific to Pex5p, Pex12p, Pex13p, or Pex19p (Fig. 2) did not reach adulthood and were still at the L1, L2, or L3 larval stage. On the other hand, at the same point of time, large numbers of adults and eggs were found in the offspring of worms that had been injected with

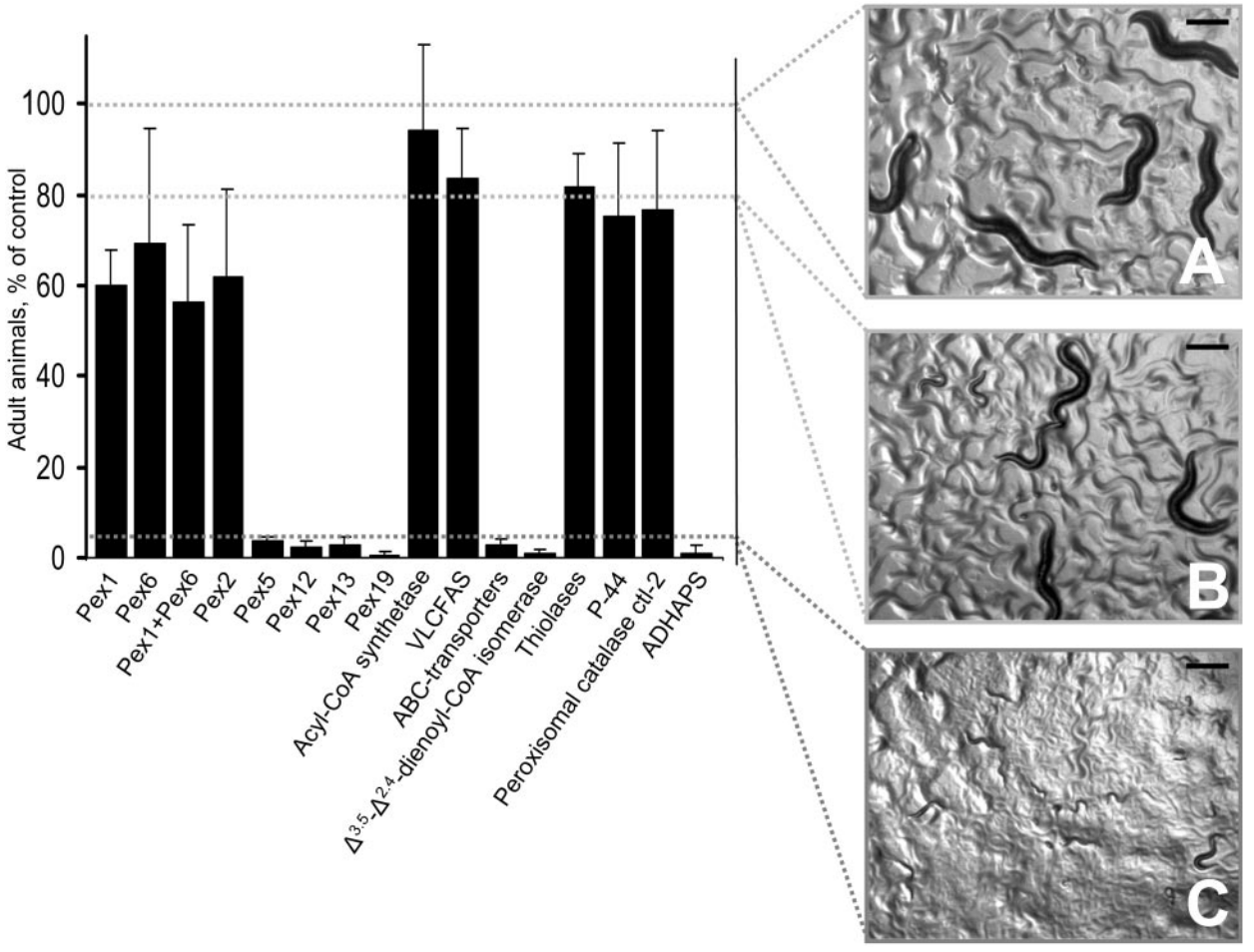

Fig. 2. Percentages of adults in the offspring, and phenotypes of F1 progeny, of worms injected with dsRNAs to target genes encoding various peroxisomal enzymes of lipid metabolism and peroxins. The percentages of adults were evaluated 3 days after injection. The percentage of adults in the offspring of control worms injected with control dsRNA was set at 100% in each independent experiment. Data from 3–7 independent experiments are presented (300–700 worms counted). Bright-field photomicrographs represent typical phenotypes and were taken 3 days postinjection. *B*: worms with slightly delayed development. *C*: worms that remain at the L1–L2 developmental stage, whereas *A* shows a control experiment in which worms reach the adult stage. Bars = 0.5 mm. ADHAPS, alkyldihydroxyacetonephosphate synthase; VLCFAs, very-long-chain fatty acids; ABC, ATP-binding cassette.

control dsRNA or dsRNAs specific to the genes encoding Pex1p, Pex6p, Pex1p, and Pex6p, or Pex2p (Fig. 2).

Chimera CFP-SKL Shows Differences in Peroxisome Size and Number in Different Parts of the Worm Body

Motley and coworkers (39) constructed fusions of GFP to either a PTS1 or a PTS2 to morphologically define peroxisomes within the cells of *C. elegans*. We have used a similar approach and have integrated an extrachromosomal array encoding a CFP-SKL fusion protein into the genome of *C. elegans* to monitor the peroxisomal import of PTS1-targeted matrix proteins and to evaluate the size, number, and morphology of peroxisomes in various dsRNA-injected worms. The integrated construct was expressed under the *let-858* promoter, which is active in all tissues and at all stages of *C. elegans* development (**http://www.ciwemb.edu/pages/firelab.html**). Expression of this fusion protein in worms produced a punctate pattern of fluorescence characteristic of peroxisomes and which can be visualized in the living organism by fluorescent microscopy (Fig. 4). The peroxisomal targeting of CFP-SKL was confirmed by double-labeling, immunofluorescence microscopy with antibodies to the well-established marker of peroxisomes, P-44 thiolase type 2, and to CFP (Fig. 5). The punctate fluorescence patterns generated by antibodies to thiolase and to CFP were superimposable and characteristic of peroxisomes (Fig. 5).

Visualization of peroxisomes in vivo using CFP-SKL unexpectedly revealed considerable differences in peroxisome size and number between various parts of the worm body. Whereas the head and tail contained reproducibly high numbers of peroxisomes of uniform size, the gut, middle body muscle, and hypodermic cells showed highly variable numbers of peroxisomes, ranging from a few to several hundred organelles. Peroxisomes in the adult worm were smaller and fewer in

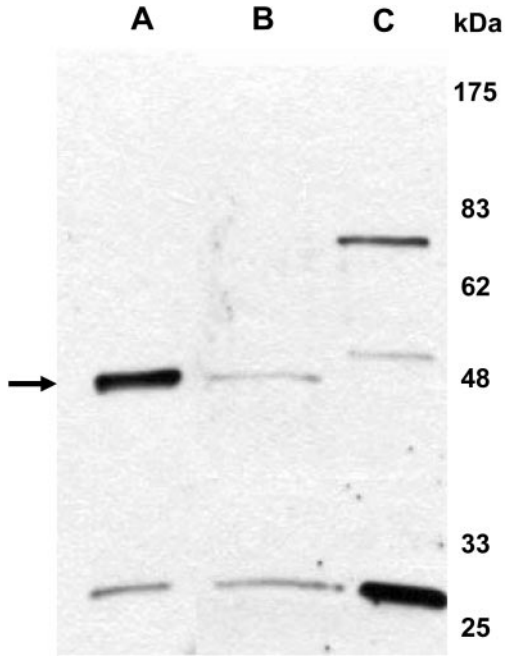

Fig. 3. RNAi reduction of the levels of type 2 thiolase P-44. Lysates of worms injected with control dsRNA (*lane A*) and worms injected with dsRNA specific to the ORF Y57A10C.6 for thiolase P-44 (*lane B*) and *E. coli* OP50 (a food source for *C. elegans*) (*lane C*) were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with anti-thiolase P-44 antibodies. Equal amounts of protein were loaded in *lanes A* and *B*. The position of migration of P-44 is designated by the arrow. The numbers at right indicate the migrations of molecular mass standards (in kDa).

number than peroxisomes in worms at various larval stages. Remarkably, only a diffuse pattern of fluorescence was seen in seam cells, one of the groups of cells from which the hypodermis forms, suggesting a lack of peroxisomes in these cells (Fig. 5*A*).

In worms injected with dsRNAs specific to the ORFs encoding Pex5p (Fig. 4*E*), Pex13p (Fig. 4*H*), or Pex19p (Fig. 4*I*), a diffuse pattern of fluorescence of CFP-SKL, characteristic of the cytosol, was observed. Therefore, these worms are deficient in the peroxisomal import of PTS1-targeted matrix proteins. In contrast, injection of dsRNAs specific to ORFs encoding the other peroxins or the other peroxisomal enzymes did not compromise the punctate fluorescence pattern of CFP-SKL characteristic of peroxisomes (Fig. 4).

It should be noted that the size and morphology of peroxisomes were significantly altered in worms injected with dsRNA to Pex12p, P-44 (type 2 peroxisomal thiolase), or to the three type 1 thiolases. Posttranscriptional silencing of the *PEX12* gene in RNAi experiments caused the accumulation of fewer peroxisomes that were significantly larger than those found in worms injected with control dsRNA (compare Fig. 4, *K* and *L*). Moreover, whereas peroxisomes in worms injected with control dsRNA were round in shape, of uniform size, and randomly distributed in the organism, the injection of dsRNA to P-44 caused the accumulation of clusters of peroxisomes that were fewer in number, irregular in shape, and of variable size, with most peroxisomes being larger than those found in worms injected with control dsRNA (Fig. 4*M*). A similar phenomenon has been observed in human, mammalian, and yeast mutant cells deficient in other enzymes of β-oxidation. In these mutant cells, loss of the enzymatic activity of acyl-CoA oxidase (5, 11, 67), fatty acyl-CoA synthetase (67), and/or another peroxisomal β-oxidation enzyme, 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase (5, 49), resulted in significant changes in peroxisome size and/or number. The primary targets for this so-called metabolic control of peroxisome abundance (5) are likely the levels of other peroxisomal β-oxidation enzymes that are dramatically increased by the loss of acyl-CoA oxidase (11) or 2-enoyl-CoA hydratase/D-3-hydroxyacyl-CoA dehydrogenase (49) enzymatic activity. The resultant overproduction of abundant matrix proteins leads to a significant change in peroxisome size (5, 49, 67) and/or number (11, 49).

DISCUSSION

Peroxisomal Metabolic Functions and Peroxins Influence the Development of C. elegans

RNAi, using dsRNA specific to a particular target locus, is now routinely employed to determine the likely loss-of-function phenotype of a gene (3). The specificity and potency of RNAi make it ideal for investigating gene function starting only with genomic sequence (16). In this study, we used RNAi to evaluate the roles for various peroxisomal enzymes and peroxins in peroxisome function and biogenesis in the nematode *C. elegans* and to compare these roles to those established for the homologous proteins in classic systems such as yeast and human. We also define the peroxisomal metabolic functions and peroxins essential for normal development of *C. elegans*. Our data suggest that the nematode model has unique features and, within certain parameters, can be employed as a powerful tool with which to study the molecular defects underlying the human peroxisomal disorders.

Injection of dsRNAs specific to the ORFs encoding the three *C. elegans* orthologs of the membrane-embedded ABC half-transporters, ALDP and ALDRP, caused a substantial delay in the development of the nematode. ALDP has been implicated in the transport of VLCFA-CoAs across the peroxisomal membrane (71), and a similar role has been suggested for other ABC half-transporters (10). A defect in ALDP in humans causes developmental delay and severe neurological deficits and leads to the most common peroxisomal disease, X-ALD (46, 70, 71). Another peroxisome-associated metabolic process, which is essential for the normal development of *C. elegans*, involves an auxiliary metabolic pathway that allows polyunsaturated fatty acids to enter the normal β-oxidation spiral. In fact, injection of dsRNA specific to the ORF encoding the first enzyme in this auxiliary pathway, $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase, caused a substantial delay in development of the nematode. In humans, an inborn

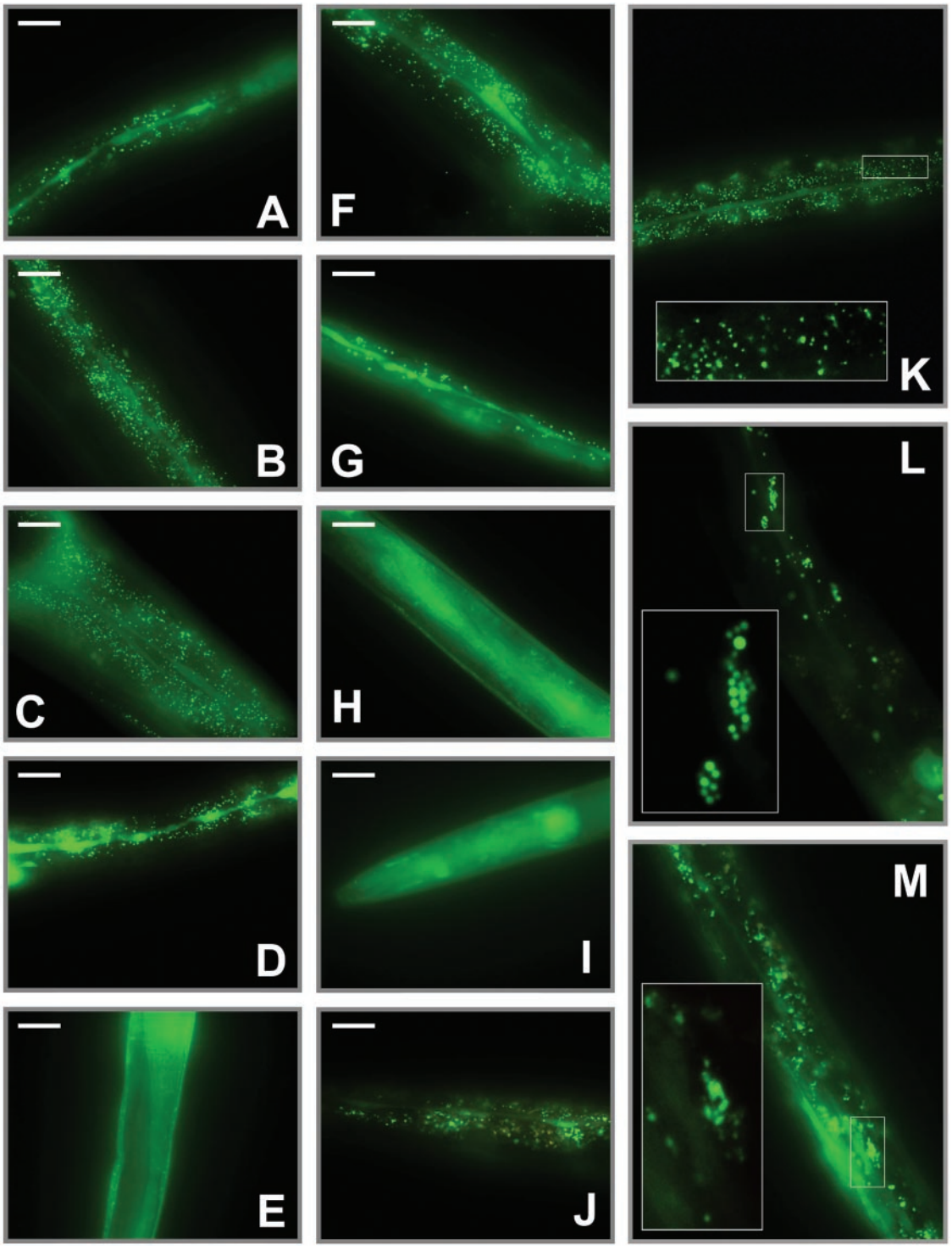

Fig. 4. The pattern of fluorescence generated by the CFP-SKL chimera. Worms injected with control (*A*), Pex1p (*B*), Pex6p (*C*), Pex1p and Pex6p (*D*), Pex5p (*E*), Pex2p (*F*), Pex12p (*G*), Pex13p (*H*), Pex19p (*I*), and the three type 1 and P-44 thiolases (*J*) dsRNAs are shown. The punctate pattern of fluorescence is characteristic of peroxisomes, while the diffuse pattern of fluorescence is characteristic of the cytosol. Bars = 20 μm. The size and morphology of peroxisomes are dramatically altered in worms injected with dsRNA to Pex12p or to P-44 (type 2 peroxisomal thiolase). Worms injected with Pex12p-dsRNA (*L*) accumulate peroxisomes that are fewer in number and of much larger size than those found in worms injected with control dsRNA (*K*). The injection of P-44-dsRNA (*M*) causes the accumulation of clusters of fewer irregularly shaped peroxisomes of variable size, most of which are larger than the round, uniformly sized and randomly distributed peroxisomes found in worms injected with control dsRNA (*K*).

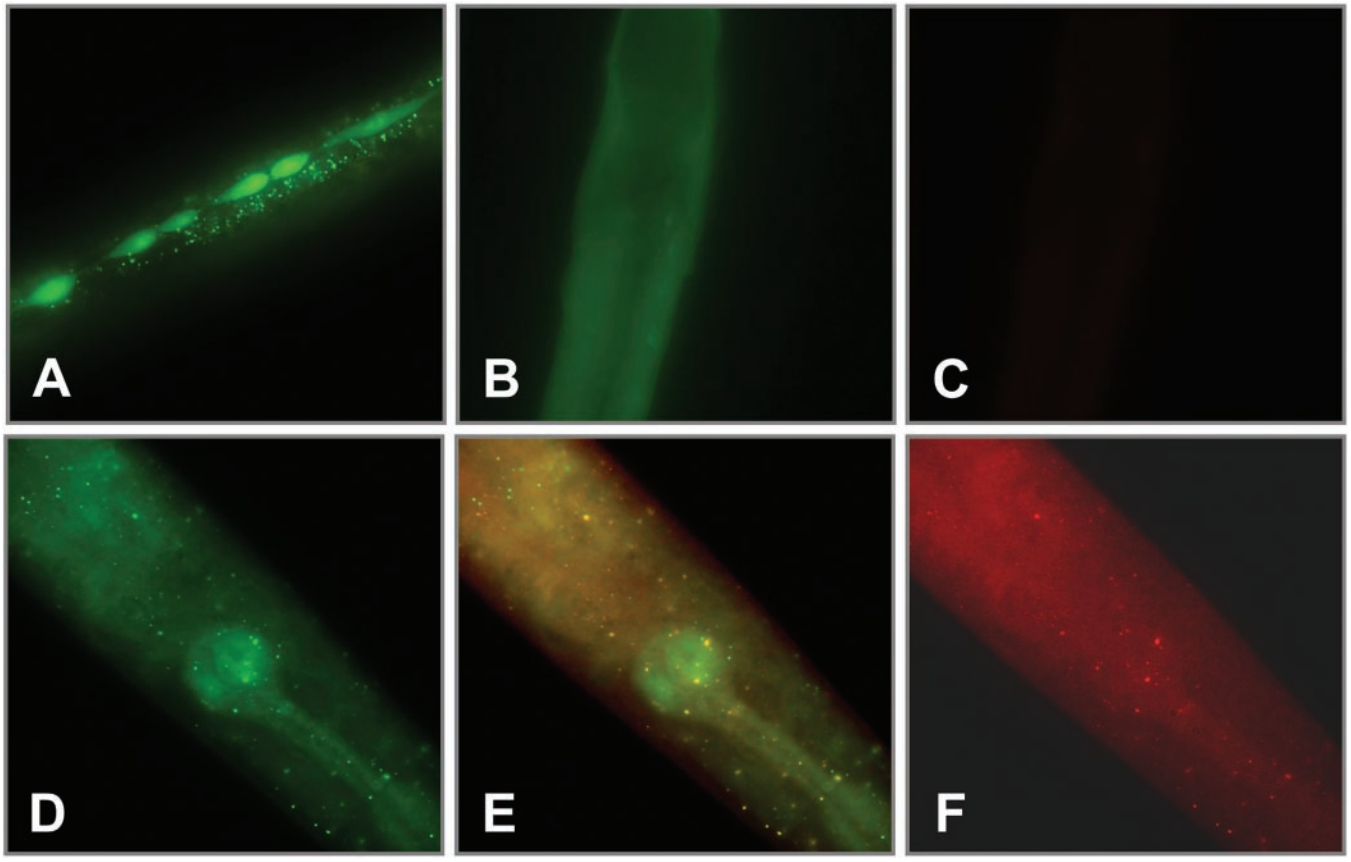

Fig. 5. Localization of CFP-SKL in seam cells, and double-labeling immunofluorescence microscopy of worms expressing CFP-SKL using anti-thiolase and anti-GFP antibodies. CFP-SKL shows a diffuse pattern of fluorescence in seam cells, suggesting a lack of peroxisomes in these cells (*A*). Anti-thiolase (*D*) and anti-GFP (*F*) antibodies were used for immunofluorescence microscopy. The merged image of *D* and *F* is shown in *E*. *B* and *C*: the fluorescence patterns generated by preimmune sera. GFP, green fluorescent protein.

error of the auxiliary pathway for the β-oxidation of fatty acids causes a peroxisomal disorder (45). The third peroxisomal metabolic process, which, according to the data of RNAi analysis presented here (Fig. 2) and elsewhere by Motley and coworkers (39), is required for the normal development of *C. elegans*, involves the biosynthesis of plasmalogen. RNAi experiments with dsRNA to ADHAPS, which catalyzes the second step in plasmalogen biosynthesis, showed a substantial delay in the development of the nematode. Likewise, in humans, inborn defects in ADHAPS cause abnormal psychomotor development and result in a lethal peroxisomal disorder, RCDP type 3 (7, 9).

Surprisingly, the phenotypes of worms treated with dsRNA to inactivate the processes of acyl-CoA synthetase-mediated activation of fatty acids, α- and β-oxidation of fatty acids, and intraperoxisomal decomposition of harmful hydrogen peroxide were close to that of control worms (Fig. 6). These results may suggest that, unlike in humans or yeast, these biochemical functions of peroxisomes may not be of major importance for the development of *C. elegans* or are duplicated by cytosolic (e.g., the cytosolic form of catalase) or mitochondrial (e.g., mitochondrial forms of thiolase) orthologs. However, as with all RNAi experiments, there is the strong possibility that posttranscriptional silencing may not have reduced the expression of some genes below a threshold necessary to display a phenotype. Immunoblot analysis with antibodies specific to the carboxyl terminus of type 2 thiolase P-44 demonstrated that the level of this protein was decreased more than 10-fold following injection of dsRNA specific for this gene (Fig. 3). Nevertheless, the observed substantial delay in nematode development caused by reductions in the activities of the ABC half-transporters, an auxiliary reaction for the β-oxidation of polyunsaturated fatty acids, and the biosynthesis of plasmalogen is specific; that is, it is not due to a general defect in the overall metabolic functioning of peroxisomes.

Human ABC half-transporters, including ALDP and ALDRP (46), and their *C. elegans* counterparts are PMPs. In *C. elegans*, ADHAPS is targeted to peroxisomes by a PTS1 (8, 39). Our study shows that these proteins are required for the normal development of *C. elegans*. In light of this, it is not surprising that deficiencies in the cytosolic shuttling receptors for the peroxisomal sorting of PMPs and PTS1-targeted proteins (Pex19p and Pex5p, respectively), as well as de-

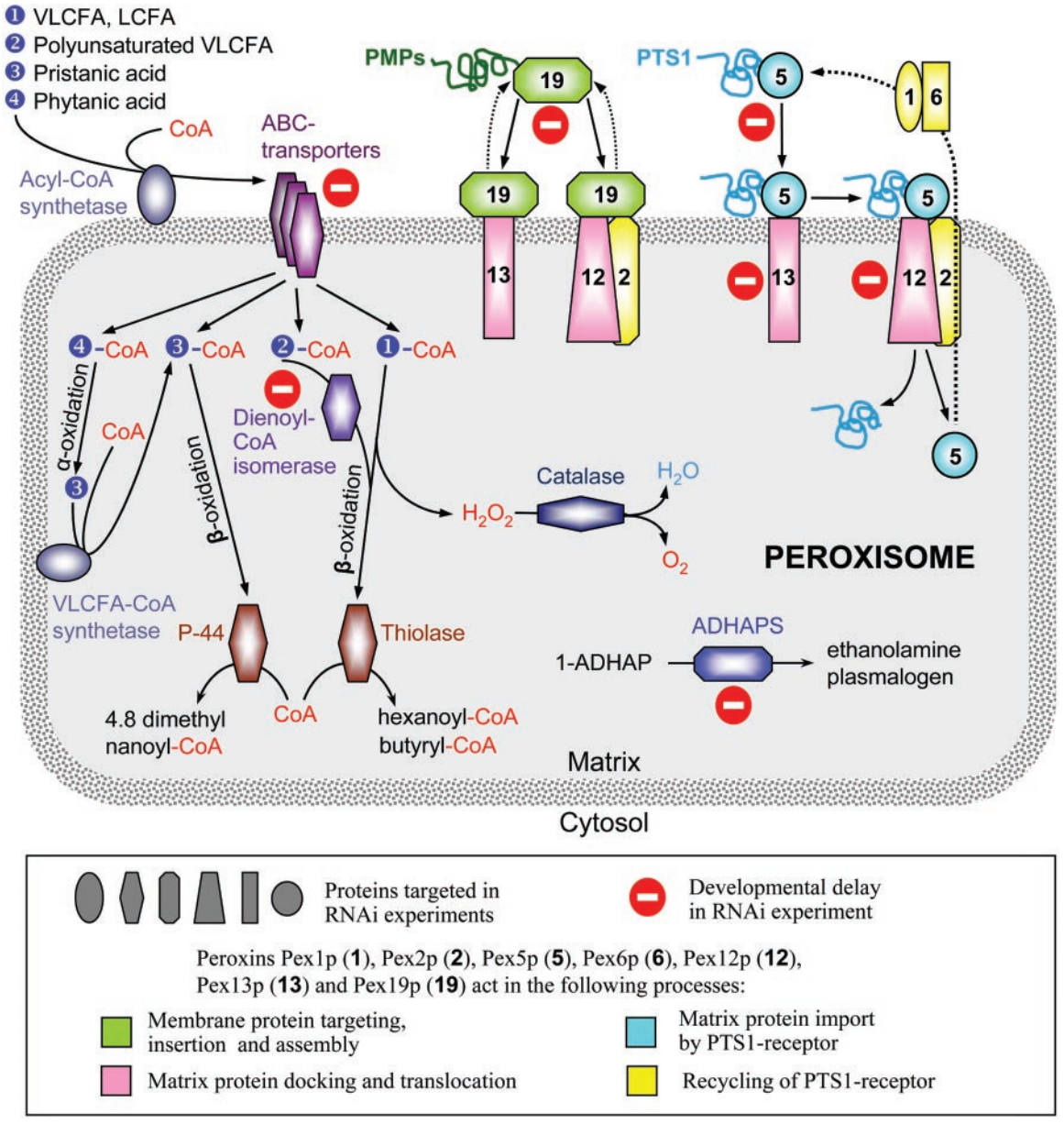

Fig. 6. Peroxisomal metabolic pathways and peroxins in *C. elegans* development.

ficiencies in the components of the docking and translocation machineries for PTS1-containing matrix proteins (Pex13p and Pex12p, respectively), are essential for the development of *C. elegans* (Fig. 6). On the other hand, we unexpectedly found that under our experimental conditions of RNAi transcriptional silencing, neither the development of the nematode nor the peroxisomal import of PTS1-targeted matrix proteins in this organism appears to require the peroxins Pex1p, Pex2p, and Pex6p. However, in all other organisms studied so far, these peroxins are essential for the peroxisomal import of matrix proteins (55, 64) and, in human cells, are required for the recycling of the PTS1 receptor to the cytosol (17, 46). In explaining this surprising observation, one should consider the fact that the PTS2 pathway for the peroxisomal import of matrix proteins, which had previously been found in all organisms studied, has been shown to be absent in *C. elegans* (39). Moreover, proteins that in all other studied organisms are targeted to the peroxisome by a PTS2, have acquired a PTS1 in *C. elegans* (18, 39). It is possible that, due to the lack of a PTS2 import machinery and the great abundance of PTS1-targeted proteins, the levels of the cytosolic shuttling receptor Pex5p and/or other components of the PTS1-dependent import machinery may be significantly higher in *C. elegans* than in other organisms. Therefore, Pex1p-, Pex2p-, and Pex6p-dependent recycling of the PTS1 receptor in the nematode may not be essential for the process of matrix protein import. Experimental confirmation of this hypothesis awaits an evaluation of the efficiency of peroxisomal protein import in Pex1p-,

Pex2p-, and Pex6p-deficient worms in which the expression levels of Pex5p and/or other components of the peroxisomal targeting/docking/translocation machinery could be precisely manipulated and varied over a wide range. Nevertheless, it must be emphasized that it would be premature at this time to dismiss a requirement for Pex1p, Pex2p, and Pex6p in the normal development of *C. elegans*.

C. elegans as a Model System for the Human Peroxisomal Disorders

This study shows that *C. elegans* can be considered as a valuable model system with which to study the molecular defects underlying the human peroxisomal disorders and to tailor therapeutics for their treatment. In fact, the developmental delay in worms injected with dsRNAs to the ABC half-transporters, $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase and ADHAPS, mimics the global developmental delay seen in patients suffering from X-ALD (46, 70, 71), a deficiency in the auxiliary pathway for the β-oxidation of polyunsaturated fatty acids (45), and RCDP type 3 (7, 9), respectively. Furthermore, the developmental delay observed in Zellweger spectrum patients afflicted with ZS, NALD, and IRD is due to defects in the peroxins Pex5p, Pex12p, Pex13p, and Pex19p (13, 17, 55). Likewise, this study shows that injection of *C. elegans* with dsRNAs to Pex5p-, Pex12p-, Pex13p-, and Pex19p caused a substantial delay in the development of the nematode.

Although the clinical phenotypes of the various peroxisomal disorders (38) and some aspects of peroxisome biogenesis in cultured fibroblasts of PBD patients (17) are well studied, the precise physiological role of peroxisomes in the normal development of a multicellular organism remains largely unknown. From this perspective, *C. elegans* provides several important advantages as a model system. First, the nematode is a genetically tractable organism with a sequenced genome and a well-studied lifecycle (2, 25). Second, *C. elegans* has a relatively simple multicellular organization (24, 31). The pedigrees of cells have been established from egg to adult organism (24, 26). Moreover, the transparent cuticle of the worm permits visualization of all cells, fluorescently tagged proteins and vital dyes within the live worm. Third, since the cell lineage, location, and synaptic connectivity of the *C. elegans* nervous system have been completely described (2, 59), *C. elegans* should prove extremely useful to analyze the molecular basis underlying the impaired neuronal development and neurodegeneration observed in patients with peroxisomal disorders (46, 68, 70, 71). Last, although studies with cultured human fibroblasts have opened up the option of pre- or postnatal diagnosis of peroxisomal disorders (69), they have not yet provided any prospect of therapy for these disorders. On the other hand, *C. elegans* has been extensively used for the testing of various pharmaceutical agents and for drug discovery (44, 59). Therefore, generation of *C. elegans* knockout mutants carrying deletions in genes that are described in this paper is the next step for unveiling the molecular causes of human peroxisomal disorders and for high-throughput screening of pharmaceuticals for their treatment.

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