EMBO reports

scientific report

Caenorhabditis elegans has a single pathway to target matrix proteins to peroxisomes

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Received April 6, 2000; revised May 11, 2000; accepted May 16, 2000

All eukaryotes so far studied, including animals, plants, yeasts and trypanosomes, have two pathways to target proteins to peroxisomes. These two pathways are specific for the two types of peroxisome targeting signal (PTS) present on peroxisomal matrix proteins. Remarkably, the complete genome sequence of *Caenorhabditis elegans* lacks the genes encoding proteins specific for the PTS2 targeting pathway. Here we show, by expression of green fluorescent protein (GFP) reporters for both pathways, that the PTS2 pathway is indeed absent in *C. elegans*. Lack of this pathway in man causes severe disease due to mislocalization of PTS2-containing proteins. This raises the question as to how *C. elegans* has accommodated the absence of the PTS2 pathway. We found by *in silico* analysis that *C. elegans* orthologues of PTS2-containing proteins have acquired a PTS1. We propose that switching of targeting signals has allowed the PTS2 pathway to be lost in the phylogenetic lineage leading to *C. elegans*.

INTRODUCTION

Proteins are targeted to peroxisomes via one of two different routes corresponding to the two types of peroxisome targeting signal (PTS). Most peroxisomal matrix proteins contain a PTS1, which consists of a somewhat degenerate C-terminal tripeptide. The original PTS1 consensus comprises (S/C/A)(K/R/H)(L) (Gould *et al.*, 1989), but many tripeptides with a two-out-of-three fit with this consensus can target proteins to peroxisomes (Sommer *et al.*, 1992; Motley *et al.*, 1995; Elgersma *et al.*, 1996; Mullen *et al.*, 1997). The PTS2 is found in only a few peroxisomal proteins and is an N-terminally located, bipartite amino acid motif, the consensus of which comprises (R)(L/V/I)X5(H/Q)(L/A) (Osumi *et al.*, 1992; Gietl, 1994; Glover *et al.*, 1994; Tsukamoto *et al.*, 1994; Kato *et al.*, 1996; Flynn *et al.*, 1998). Newly synthesized peroxisomal matrix proteins are recognized by cytosolic receptors specific for either PTS1 or PTS2. These receptors target the PTS-containing proteins to peroxisomes, where the two pathways converge and make use of a common translocation machinery (for reviews see Subramani, 1998; Hettema *et al.*, 1999). Both protein targeting pathways have been identified in highly divergent eukaryotes, including trypanosomes, yeasts, plants and animals. The presence of a PTS2 pathway in such highly divergent eukaryotes implies that both PTS1 and PTS2 targeting pathways were present in the ancestral eukaryotic cell.

Three proteins, Pex7p (Marzioch *et al.*, 1994; Zhang and Lazarow, 1995), Pex18p and Pex21p (Purdue *et al.*, 1998), are required specifically for targeting PTS2 proteins to peroxisomes in *Saccharomyces cerevisiae*. Of these three proteins, the human PTS2 receptor protein Pex7p could be identified by similarity to the yeast receptor (Braverman *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997). Orthologues of Pex18p and Pex21p have not been found. Cells lacking Pex7p mistarget PTS2-containing proteins to the cytoplasm. In man, this mislocalization results in loss of activity of the PTS2-containing enzyme alkyldihydroxyacetonephosphate synthase (ADHAPS), causing the severe disorder rhizomelic chondrodysplasia punctata (RCDP) (Wanders *et al.*, 1994; de Vet *et al.*, 1998a). We were therefore surprised to find that orthologues of proteins required in the PTS2-targeting pathway are absent from the *Caenorhabditis elegans* genome databases.

Here we show, by expression of green fluorescent protein (GFP) reporter proteins, that the PTS2-specific targeting pathway is indeed absent in *C. elegans*. Furthermore, identification of *C. elegans* orthologues of PTS2-containing proteins shows that these proteins no longer have a PTS2, but have acquired a PTS1.

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We also found that ADHAPS, which has undergone targeting signal switching in *C. elegans* (de Vet *et al.*, 1998b) and is required for normal development in man (Wanders *et al.*, 1994; de Vet *et al.*, 1998a), is also required for normal development in *C. elegans*. Because loss of the PTS2 targeting pathway (resulting in loss of ADHAPS activity) before rerouting of ADHAPS to the PTS1 pathway would have severe consequences for the survival of any individuals in which this occurred, we propose that the PTS2 targeting pathway must have been lost secondarily to targeting signal switching of at least ADHAPS.

RESULTS AND DISCUSSION

The PTS2 pathway is absent in *C. elegans*

We searched the *C. elegans* genome databases for any of the three proteins (Pex7p, Pex18p and Pex21p) required specifically for targeting PTS2-containing proteins to peroxisomes in *S. cerevisiae*. We did not find any orthologues for these peroxins, although we readily identified putative *C. elegans* orthologues of many other peroxisomal proteins including the PTS1 receptor. We were also able to identify putative Pex7p orthologues in divergent organisms such as *Drosophila melanogaster* (DDBJ/EMBL/GenBank accession No. AAF50379.1), *Dictyostelium discoideum* (slime mould, accession No. C257251.1) and *Arabidopsis thaliana* (accession No. AAD27848). The absence of components required specifically in the PTS2 targeting pathway was corroborated when we searched the *C. elegans* database for the PTS2-containing proteins themselves. 3-Ketoacyl-CoA thiolase contains a PTS2 in all organisms tested, but in *C. elegans* three candidate orthologues of this protein all contain a PTS1 (Table I). de Vet *et al.* (1998b) reported that ADHAPS, which contains a PTS2 in mammals (de Vet *et al.*, 1997), contains a PTS1 in *C. elegans*. Phytanoyl-CoA hydroxylase also has a PTS2 in mammals and a PTS1 in *C. elegans* (Table I). Indeed, putative *C. elegans* orthologues of PTS2-containing proteins all contain a PTS1 (Table I). These observations suggested the complete absence of the PTS2 targeting pathway. We tested this *in vivo* by expression in *C. elegans* of GFP reporters for the two targeting pathways.

We used the presequence of rat 3-ketoacyl-CoA thiolase fused to GFP as a reporter protein for PTS2 import. Rat thiolase PTS2 has previously been shown to import a reporter protein into peroxisomes of plants (Flynn *et al.*, 1998) and trypanosomes (Blattner *et al.*, 1995). In *C. elegans*, however, a diffuse pattern of staining is seen after expression of PTS2–GFP (Figure 1F). The lack of import into peroxisomes indicates the absence of this targeting route in *C. elegans*. As a control, we expressed the reporter for the PTS1 pathway: GFP–PTS1 produces the punctate labelling pattern characteristic of peroxisomes (Figure 1E). We also expressed the two GFP reporters in such highly divergent cells as *S. cerevisiae* and human fibroblasts. Figure 1A–D shows the punctate pattern of staining indicating import into peroxisomes. We conclude that the PTS2 targeting pathway is absent in *C. elegans.*

ADHAPS is required for normal growth/development of *C. elegans*

In humans, three PTS2-containing proteins are currently known (Table I). In humans, loss of the PTS2 receptor, Pex7p, causes multiple enzyme deficiencies due to missorting of (at least) three (PTS2-containing) proteins, and results in the severe developmental disorder RCDP (Braverman *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997). It is the mislocalization and resulting loss of activity (Biermann *et al.*, 1999) of one of these PTS2-containing enzymes, ADHAPS, that gives rise to the disease; in some RCDP patients, the only deficient activity is that of ADHAPS (Wanders *et al.*, 1994) as a result of mutations in the gene encoding this enzyme (de Vet *et al.*, 1998a).

We wanted to determine whether ADHAPS is important for normal development of *C. elegans.* We inhibited the function of ADHAPS in *C. elegans* using RNA interference (Fire *et al.*, 1998), a technique whereby double-stranded (ds)RNA of a target gene is injected into the gonads of adult hermaphrodites, resulting in strongly reduced activity of the target gene in the offspring of the injected animals. As shown in Figure 2, the growth of the offspring of ADHAPS-dsRNA-injected worms was strongly retarded compared with the growth of offspring of worms injected with a control dsRNA. Eight days after the eggs of the injected worms had hatched, the control offspring had completely cleared the bacterial lawn (on which they feed), and many eggs, larvae and adults were visible, indicating that the offspring of the injected worms had completed at least one life cycle. In contrast, the offspring of the ADHAPS-dsRNA-injected worms were still larval size, none of these worms had reached adulthood and no eggs were visible. The bacterial lawn was still intact on these plates. We conclude that ADHAPS is required for normal growth/development of *C. elegans*.

How did *C. elegans* lose the PTS2 route for protein targeting?

We propose that targeting signal switching from PTS2 to PTS1 must have occurred before loss of the PTS2-specific targeting pathway, as proper localization of peroxisomal enzymes is required for their biological activity, and loss of their function is a strongly disadvantageous event, as we have shown for *C. elegans* ADHAPS. Indeed, we think that proteins might acquire a PTS1 easily as this signal is highly degenerate and consists only of a C-terminal tripeptide. The acquisition of a PTS1 by a PTS2 protein could subsequently allow loss of the PTS2 without affecting its subcellular localization. Indeed, variation between possession of a PTS1 or a PTS2 does occur in peroxisomal matrix proteins in rare instances (see Table I). Targeting signals are usually found in N- or C-terminal extensions that crystallographic studies show protrude from the protein. Mutations in these extensions would not compromise the enzymatic activity of the protein, and may occasionally create a functional targeting signal. A striking example is alanine:glyoxalate aminotransferase I, which has changed its subcellular location between peroxisomes, mitochondria and cytoplasm several times during the evolution of mammals (Danpure, 1997). By analogy, *C. elegans* may have switched its PTS2 proteins to PTS1 proteins.

It remains a possibility that in the specific case of ADHAPS, the ancestral form had a PTS1 and acquired a PTS2 in the lineage leading to the chordates, as we found ADHAPS to have a PTS1 in all organisms except zebra fish and mammals, in which it has a PTS2 (Table I). However, we favour the reverse scenario for the reasons described above.

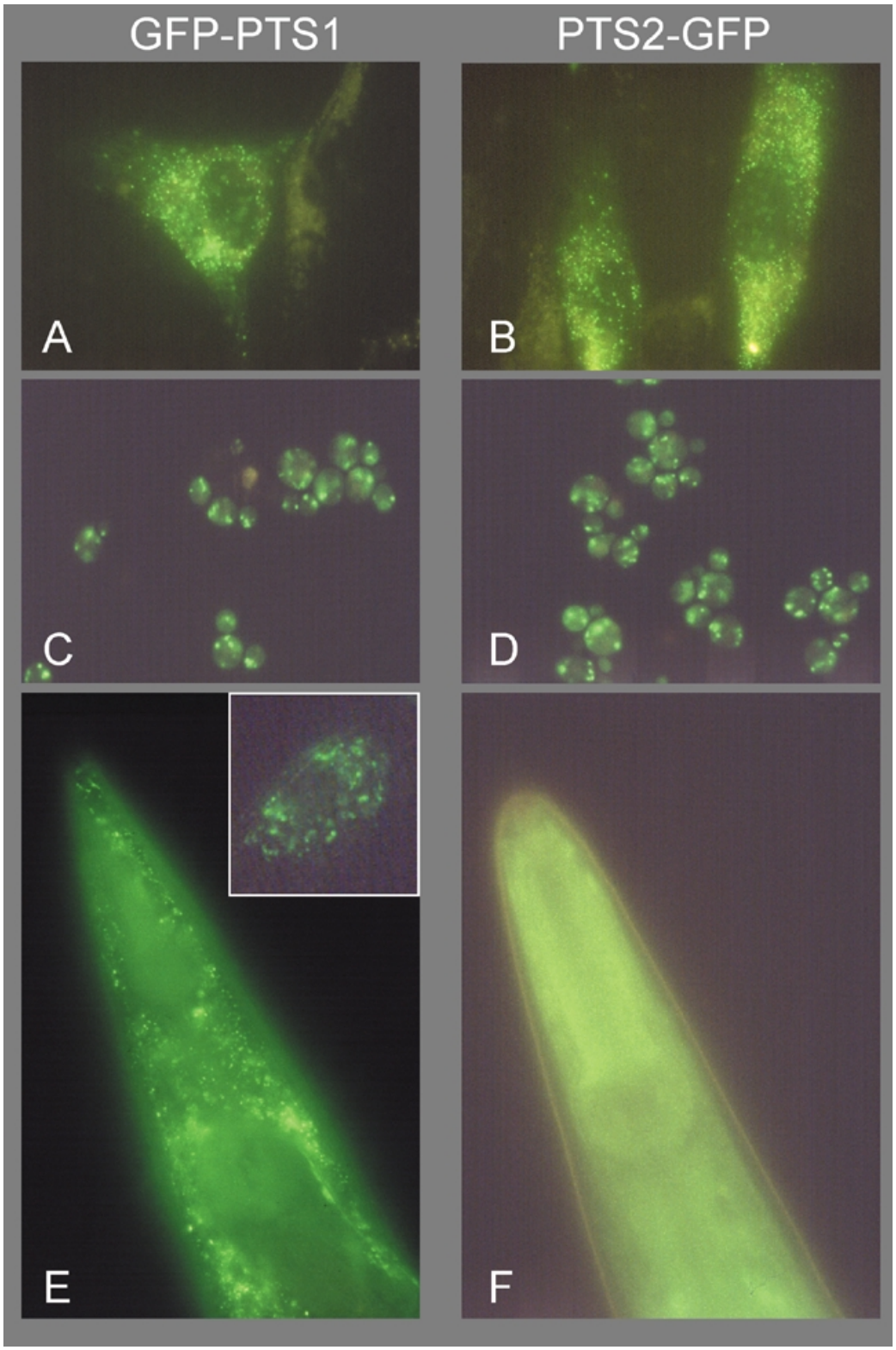

Fig. 1. PTS2–GFP is not imported into peroxisomes in *C. elegans.* GFP–PTS1 (left panels) and PTS2–GFP (right panels) are imported into peroxisomes in human fibroblasts (**A** and **B**) and *S. cerevisiae* (**C** and **D**). Expression of GFP–PTS1 in *C. elegans* (**E**) shows a punctate labelling pattern indicative of peroxisome labelling. Inset: a single *C. elegans* cell in a backgound of GFP-negative cells showing punctate fluorescence. Single fluorescent cells can be observed with a low frequency in *C. elegans* carrying this GFP–PTS1 reporter transgene due to loss of the extrachromosomal plasmid array carrying the transgene. Expression of PTS2–GFP in *C. elegans* (**F**). A diffuse pattern of labelling after expression of PTS2–GFP is also seen in yeast (not shown) and human cells (Motley *et al*., 1997) lacking Pex7p.

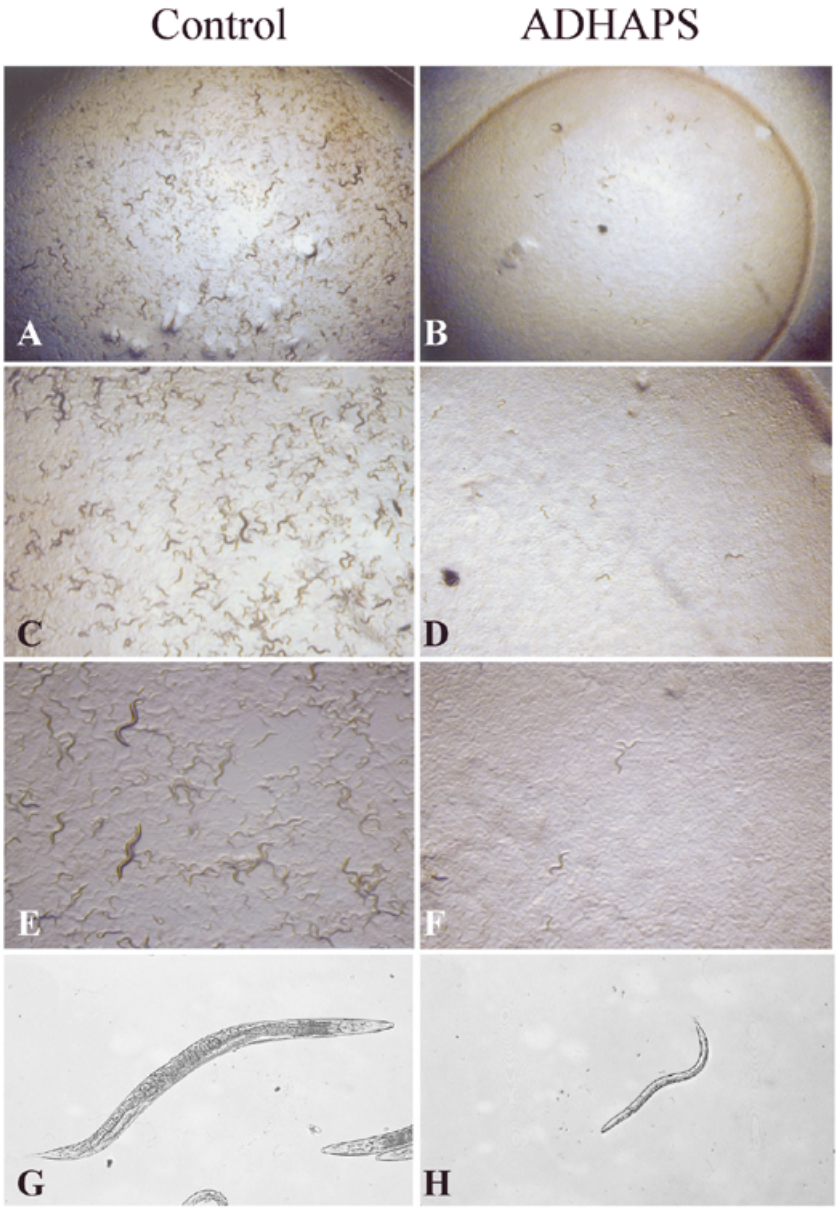

Fig. 2. ADHAPS is required for normal growth/development of *C. elegans*. Offspring of dsRNA-injected worms are shown 8 days after hatching, at increasing magnification. In the control panels, the animals had cleared the bacterial lawn (A), and there were large numbers of eggs, larvae and adults (**A**, **C** and **E**), indicating that the offspring of control-dsRNA-injected worms had completed at least one life cycle. The offspring of ADHAPS-dsRNA-injected worms (right-hand panels) were still larval sized at this time point, and no adult worms were visible (**B**, **D** and **F**). Also, the bacterial lawn was still intact (B). The highest magnification (G and H) shows offspring of (**G**) a control-dsRNA-injected worm (~1.5 mm in length) and (**H**) an ADHAPS-dsRNA-injected worm of the same age.

In summary, we propose that the surprisingly high degeneracy of PTS1 (Gould *et al.*, 1989; Sommer *et al.*, 1992; Motley *et al.*, 1995; Elgersma *et al.*, 1996; Mullen *et al.*, 1997) allows switching from PTS2 to PTS1. Eventually, this could have led to switching of all PTS2-containing proteins to PTS1-containing proteins, and a subsequent loss of the PTS2 import machinery. With more eukaryotic genome sequences becoming available, it will be interesting to observe whether other organisms lacking a PTS2 targeting pathway will be identified. Indeed, it is intriguing that although *D. melanogaster* has a candidate PTS2 receptor, *D. melanogaster* ADHAPS and three *D. melanogaster* thiolase orthologues all contain a PTS1 rather than a PTS2. A very striking result of the recent molecular based reorganization of the metazoan phylogenetic tree has placed nematodes as a sister group of arthropods (for a perspective see Adoutte *et al.*, 2000). Perhaps the targeting signal switching occurred in the common lineage leading to *C. elegans* and *D. melanogaster*, and *D. melanogaster* has yet to lose all remnants of the PTS2 targeting pathway,

Table I. Peroxisomal proteins that have undergone targeting signal switches

Peroxisomal protein	Species	PTS1 consensus	PTS2 consensus	Accession No.	PTS1[a] of *C. elegans* orthologue
3-Ketoacyl-CoA thiolase	*D. discoidium*	–	+	AU060090	
	yeasts	–	+[b]		
	plants	–	+[b]		
	mammals	–	+[b]		
	C. elegans	+	–	T02G5.8[c]	-QKL
				T02G5.7	-KKL
				T02G5.4	-QKL
ADHAPS	*T. brucei*	+	–	AAD19697	
	D. discoidium	+	–	CAA09333	
	zebra fish	–	+	AI667604	
	mammals	–	+[b]		
	C. elegans	+	–	CAA05690.1	-CKL
Phytanoyl-CoA hydroxylase	mammals	–	+[b]		
	C. elegans	+	–	yk151d7.3	-ANL
Peroxisomal malate dehydrogenase	*S. cerevisiae*	+[b]	–		
	plants	–	+[b]		
Peroxisomal citrate synthase	*S. cerevisiae*	+[b]	–		
	plants	–	+[b]		
Acyl-CoA oxidase	*Pichia pastoris*	+[b]	–		
	plants	–	+[b]		
	mammals	+[b]	–		
	C. elegans	+	–	C48B4.1	-SKL
				F58F9.7	-SKL
				F59F4.1	-SKL
				F08A8.3	-AKL
				F08A8.2	-AKL
				F08A8.1	-SKL
				F08A8.4	-AKL

[a]The C-termini of all the putative *C. elegans* orthologues have been shown to be functional in targeting a protein to peroxisomes in other organisms (Gould *et al*., 1989; Sommer *et al*., 1992; Motley *et al*., 1995; Elgersma *et al*., 1996; Mullen *et al*., 1997).
[b]The functionality of these PTSs has been experimentally validated.
[c]The protein encoded by this ORF is the most probable counterpart of the mammalian type I peroxisomal thiolase (Maebuchi *et al*., 1999).

whereas this has already occurred in the rapidly evolving *C. elegans*.

METHODS

Plasmids. The GFP–PTS1 reporter for expression in *C. elegans* was constructed from plasmid pPD117.01 (a gift from A. Fire), in which the gene encoding GFP contains artificial introns for optimal expression in *C. elegans*. A linker encoding amino acids $PLHSKL_{COOH}$ was ligated onto the 3′-end of GFP to create the C-terminal PTS1, and the mec7 promoter was replaced with a 1.6 kb genomic PCR fragment containing the SUR5 (house-keeping) promoter (obtained using forward primer CCGTCT-AGACGTTAGGGTGGAATTGAACCC and reverse primer GGAATCGATTCTGAAAACAAAATGTAAAGTTC) into the *Xba*I *Cla*I sites for expression in many cell types.

The PTS2–GFP expression constructs were derived from the PTS2 of rat 3-ketoacyl-CoA thiolase, a plasmid kindly provided by S. Gould. The *C. elegans* PTS2–GFP reporter (pAM8) was constructed by replacing the 5′ region (*Cla*I–*Nco*I) of GFP in pPD117.01 (a gift from A. Fire) with a PTS2–GFP PCR fragment containing the appropriate sites. The mec7 promoter was then replaced with the house-keeping SUR5 promoter as described

for the GFP–PTS1 expression construct. The yeast PTS2–GFP expression construct was constructed from the *C. elegans* GFP reporter construct pAM8: a *Cla*I–*Nco*I fragment containing the PTS2–GFP 5′-region was fused with an *Nco*I–*Hin*dIII fragment containing the remainder of the GFP open reading frame (ORF) (lacking *C. elegans* introns) into the *Acc*I–*Hin*dIII sites of the yeast episomal plasmid Yeplac181 containing the catalase A promoter region. In this way, all three PTS2–GFP fusion proteins are identical.

Expression analysis. The *C. elegans* GFP reporter plasmids (pAM7 and pAM8) were each co-injected with the dominant marker *rol-6* (pRF4) into the gonads of young hermaphrodites, and first generation transformants were examined for localization of GFP. The GFP expression constructs were introduced into yeast and human fibroblasts (Motley *et al.*, 1997) as described previously. Fluorescent micrographs were recorded on a Zeiss microscope using standard filter sets.

RNA interference experiments. RNA was produced from a PCR template carrying the T7 and T3 promoter sequences at the 5′ and 3′ ends, respectively, using the Ambion MEGAscript T7 and T3 kits following the manufacturer's instructions. The control template was amplified from *C. elegans* N2 genomic DNA (forward primer TAATACGACTCACTATAGGctttcgcgtcaaggctatc, reverse primer AATTAACCCTCACTAAAGGctcttcggcttcaccagaac, where the T7 and T3 promoter sequences are shown in upper case) and the ADHAPS template was amplified from a plasmid encoding the full-length cDNA sequence of ADHAPS (de Vet *et al.*, 1998b) kindly provided by E. de Vet. The control template contains a segment of the coding region of the gene *daf-12*, which is required for dauer formation. After RNA production using the Megascript kit, the sense and antisense strands were pooled and purified using Qiagen RNEasy columns and annealed in 6× injection buffer for 30 min at 37°C as described previously (Fire *et al.*, 1998). Formation of dsRNA was confirmed by testing the mobility of single-stranded relative to dsRNA on a standard (non-denaturing) agarose gel.

Young adult N2 hermaphrodites were injected into the gonads as described (Fire *et al.*, 1998). After recovery, injected animals were transferred to fresh culture plates at 16 h intervals. This results in a series of approximately synchronous cohorts in which it is straightforward to identify phenotypic differences. After the first 16 h interval, all the progeny of the ADHAPS-dsRNA-injected worms showed the retarded growth phenotype.

Database analyses. Candidate Pex7p orthologues were identified by TBLASTN and PSI BLAST searches in the NCBI genome databases (non-redundant, other ESTS, *D. melanogaster*) using various search parameters. Candidate orthologues were confirmed by reciprocal TBLASTN searches in the same databases. A similar approach of reciprocal BLAST searching was used to identify orthologues of PTS2-containing proteins in the NCBI and *C. elegans* genome databases.

ACKNOWLEDGEMENTS

We are grateful to The Wellcome Trust *C. elegans* course instructors, Patty Kuwabara in particular, for excellent instruction, to Edwin de Vet, Andy Fire and Steven Gould for plasmids, and to the Netherlands Organisation for Scientific Research for financial support, in the form of a fellowship to A.M.M.

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DOI: 10.1093/embo-reports/kvd010