

## Supplementary methods

### *Site-directed mutagenesis*

Site-directed mutants were generated using a QuickChange II kit (Stratagene), according to manufacturers' instructions. Primers are given in Table S2. All constructs were sequenced to verify the presence of the mutation and to confirm that no unwanted mutations had been introduced.

### *Plasmids for yeast expression*

CTS was excised from CTS/pRS416GPD (1) with *Bam* HI/*Sal* I and cloned in the corresponding sites of pEL30 and pIJL30. LACS6 was amplified from LACS6/pZL1 (2) using primer pair FT175/FT176, LACS7 was amplified from LACS7/pCRTOP0II (2), using primer pair FT177/FT178 and both were cloned into the *Kpn* I/*Xba* I sites of pEL30 and pIJL30. To mistarget LACS7 to the cytosol, the ORF was amplified with primer pair FT184/FT185, restricted with *Kpn* I/*Xba* I and cloned into the corresponding sites of pEL30. This removed PTS2 (R<sub>10</sub>LETIRSHI<sub>18</sub>), adding a new methionine start codon (adjacent to Asp19) and inserted a stop codon before the PTS1 (SKL) at Ser399.

### *Plasmids for insect cell expression*

The *CTS* ORF was amplified in two overlapping halves using primer pairs FT41/FT35 and FT36/FT45 (Table S2), with plasmid H1A6T7 (Accession number: AF378120) as template. The fragments were cloned into pCR4BluntTOPO (Invitrogen, Paisley, UK), excised with *Bam* HI-*Pst* I and *Pst* I-*Bst* EII, respectively, and cloned into the *Bam* HI-*Bst* EII sites of pBlueBac4::*MDR*-12His (kind gift of R. Callaghan, Australian National University, Canberra) in a three-way ligation. This gave pBlueBac4::*CTS*-12His. The 3' portion of *CTS* was re-amplified from pBlueBac4::*CTS*-12His using primer pairs FT36/FT46, cloned into pCR4BluntTOPO (Invitrogen, Paisley, UK), and excised with *Pst* I-*Not* I. This fragment was ligated together with the original *Bam* HI-*Pst* I fragment encoding the 5' portion of *CTS* into pBacPAK9 (Clontech, St-Germain-en-Laye, France) restricted with *Bam* HI-*Not* I to reassemble the entire *CTS* ORF plus an in-frame dodeca-histidine tag. Finally, the entire *CTS* ORF plus tag was excised from pBacPAK9-*CTS*-12His and cloned into the *Bam* HI-*Not* I sites of pFastBac1 (Invitrogen, Paisley, UK).

### *Yeast subcellular fractionation and Nycodenz gradients.*

Subcellular fractionation of oleate-grown yeast cells was performed as described by (3). Organellar pellets were layered on top of a 15 to 35% (w/v) Nycodenz gradient (12 ml), with a cushion of 1.0 ml of 50% (w/v) Nycodenz solution. All Nycodenz solutions contained 5 mM MES (morpholinoethanesulfonic acid, pH 6.0), 1 mM EDTA, 1 mM KCl, and 8.5% (w/v) sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor (MSE 8x35) at 19,000 rpm at 4°C. Fractions, were assayed for the peroxisomal marker 3-hydroxyacyl-CoA dehydrogenase was measured on a Cobas-Fara centrifugal analyzer by monitoring the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm. In addition, 150 µl aliquots were taken from the individual fractions derived each from Nycodenz gradient, to which 150 µl of Laemmli sample buffer was added followed by analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

#### *Immunoblotting of yeast extracts.*

Yeast extracts were subjected to SDS-PAGE on a 10% acrylamide gel. Proteins were transferred to nitrocellulose by semi-dry blotting, blocked with BSA and probed with affinity-purified anti-CTS polyclonal antibody (1), followed by incubation with IRDye secondary antibody (LI-COR 1 : 10,000). Visualization of the signal was performed with the Odyssey IR imaging system (LI-COR Biosciences).

#### *Generation and testing of CTS-GFP plants*

A plant transformation vector was generated by transferring the terminator region of pUC18-spGFP6 (4) to the vector pGreenII0229 (5). First, annealed oligonucleotides (5'-CTAGAGGATCCGCATG-3' and 5'-CGGATCCT-3') were ligated into the *Xba* I/*Sph* I sites of pUC18-spGFP6 to introduce a *Bam* HI site. The spacer-GFP-terminator cassette of pUC18-spGFP6 was excised with *Kpn* I (made blunt with T4 DNA polymerase [New England Biolabs]) and *Sma* I and ligated into the *Not* I (blunt-ended)/*Sma* I sites of pGreenII0229 to yield pG0229-spGFP-T. A CTS promoter-ORF cassette was prepared in several stages, as described in (6), except that the 3' portion of the CTS ORF was amplified with CTS3FW2 and CTS 3'RV w/o Stop to remove the native stop codon. Following reconstruction in pBluescript SKII, the CTS promoter-ORF cassette was excised with *Kpn* I and *Sma* I and ligated into the *Kpn* I and *Sma* I sites of pG0229-spGFP-T. Generation of homozygous lines expressing CTS-pro-ORF-sp-GFP and complementation of *cts-1* was as described (7).

#### **Supplementary methods references**

1. Nyathi Y, et al. (2010) [The Arabidopsis peroxisomal ABC transporter, comatose, complements the \*Saccharomyces cerevisiae\* \*pxa1 pxa2Δ\* mutant for metabolism of long-chain fatty acids and exhibits fatty acyl-CoA-stimulated ATPase activity.](#) *J Biol Chem* 2285:29892-29902.
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3. van der Leij, I, et al. (1992) Isolation of peroxisome assembly mutants from *Saccharomyces cerevisiae* with different morphologies using a novel positive selection procedure. *J Cell Biol* 119, 153-162.
4. Komarova NY, et al. (2012) Determinants for Arabidopsis peptide transporter targeting to the tonoplast or plasma membrane *Traffic* 13:1090-1105.
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7. Dietrich D, et al. (2009) [Mutations in the Arabidopsis peroxisomal ABC transporter COMATOSE allow differentiation between multiple functions \*in planta\*: insights from an allelic series.](#) *Mol Biol Cell* 20:530-543.
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9. van Roermund et al. 2012 [Peroxisomal Fatty Acid Uptake Mechanism in \*Saccharomyces cerevisiae\*.](#) *J Biol Chem* 287:20144-20153.

## Supplementary Figures

### Figure S1. CTS<sub>S810N</sub> is expressed in yeast peroxisomes

The *Saccharomyces cerevisiae* *pxa1/pxa2Δ* mutant was transformed with CTS<sub>S810N</sub> and subcellular fractions prepared by differential centrifugation and Nycodenz equilibrium density gradient centrifugation. A. Activity of the peroxisomal marker enzyme, 3-hydroxy-CoA dehydrogenase (3-HAD) in homogenate (H), pellet (P) and supernatant (S) fractions, expressed as a percentage of total. CTS<sub>S810N</sub> protein was detected by immunoblotting with an antibody raised to CTS (lower panel). B. 3-HAD activity and CTS<sub>S810N</sub> protein in fractions from the Nycodenz gradient.

### Figure S2. LACS 6 and 7 are functional and correctly targeted when expressed in yeast cells

The *Saccharomyces cerevisiae* *pxa1/pxa2/faa2Δ* mutant was transformed with different constructs as indicated. A: C8:0-CoA synthetase activity of detergent (Triton) lysates of oleate-grown cells. Values are means  $\pm$  SD (n=2). B: oleate-grown cells were fractionated by differential centrifugation into homogenate, 17,000.g pellet and supernatant and C8:0 acyl-CoA synthetase activity was determined. 3-hydroxy-CoA dehydrogenase (3-HAD) activity was used as a marker for peroxisomes. Data are expressed as a percentage of the total activity. The experiment was performed 3 times and a representative experiment is shown. C: the 17,000.g pellet was fractionated further by Nycodenz equilibrium density gradient centrifugation. C18:1- and C8:0-CoA synthetase activity was measured in the peroxisome-enriched fraction as identified by 3-HAD activity.

### Figure S3. CTS-GFP is present in a complex in *Arabidopsis thaliana* seedlings

A homozygous transgenic line of the null *cts-1* allele expressing CTS-GFP under the control of the native *CTS* promoter was tested for expression and function of CTS-GFP. A: CTS-GFP complements *cts-1* for germination and seedling establishment. Seeds were sterilised and plated on 0.5 X MS medium in the presence and absence of 0.5 % sucrose ( $\pm$  suc). In the lower panel, untransformed *cts-1* seedlings were induced to germinate by mechanically rupturing the seed coat. B: Membrane proteins from *cts-1::CTSpro-CTS-GFP* seedlings were solubilised with  $\beta$ -dodecyl maltoside. Solubilised membrane proteins were subjected to blue-native PAGE in (first dimension) followed by

denaturing SDS-PAGE and immunoblotting with an antibody against GFP. Positions of molecular weight markers (sizes in kDa) are shown above (first dimension) and to the left (second dimension) of the panel. The lower molecular weight band is a cleavage product containing GFP. C: Membrane proteins were solubilised from wild type Arabidopsis suspension culture cells with Triton X-100 and subjected to blue-native PAGE followed by denaturing SDS-PAGE and immunoblotting for both CTS and LACS6/7.