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Pseudo half-molecules of the ABC transporter, COMATOSE, bind Pex19 and target to peroxisomes independently but are both required for activity

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ABSTRACT

Peroxisomal ABC transporters of animals and fungi are "half-size" proteins which dimerise to form a functional transporter. However, peroxisomal ABC transporters of land plants are synthesised as a single polypeptide which represents a fused heterodimer. The N- and C-terminal pseudo-halves of COMATOSE (CTS; AtABCD1) were expressed as separate polypeptides which bound Pex19 in vitro and targeted independently to the peroxisome membrane in yeast, where they were stable but not functional. When co-expressed, the pseudo-halves were fully functional as indicated by ATPase activity and rescue of the *pxa1pxa2Δ* mutant for growth on oleate. The functional significance of heterodimer asymmetry is discussed.

Structured summary of protein interactions:
PEX19-1 binds to **CTS-N** by pull down (View Interaction)
PEX19-1 binds to **CTS-C** by pull down (View Interaction)
PEX19-2 binds to **CTS-N** by pull down (View Interaction)
PEX19-2 binds to **CTS-N** by pull down (View Interaction)

1. Introduction

ABC transporters couple ATP hydrolysis to the trans-membrane movement of a wide range of substrates. All ABC transporters comprise four functional domains: two sets of primarily α-helical transmembrane domains (TMDs) which associate to form the transport pathway for the substrate and two nucleotide binding domains (NBDs) which dimerise and form two composite ATP binding sites at the dimer interface.

Peroxisomes from all eukaryotes contain at least one member of the ABC subfamily D which transports lipophilic/amphipathic substrates from the cytosol into the peroxisome for β-oxidation. Comatose (CTS; AtABCD1, also known as AtPXA1 and PED3) is a full size peroxisomal ABC transporter from Arabidopsis with the domain organisation [TMD-NBD-TMD-NBD] [1–3]. Thus CTS can be considered to be a fused heterodimer consisting of two homologous but distinct halves. This gene structure is found in all land plants including bryophytes but is not present in algae, suggesting that this fusion happened once in the land plant lineage more than 450 Ma ago (Fig. 1). In contrast, animal and fungal peroxisomal ABC transporters are all half transporters with the organisation [TMD-NBD] that have to dimerise for activity [4]. Mammalian ABCD proteins are functional as homodimers, but in *Saccharomyces cerevisiae*, the half transporters Pxa1p and Pxa2p heterodimerise to transport long chain acyl CoAs [5,6]. We have shown recently that CTS targets to peroxisomes when expressed in oleate-grown *S. cerevisiae* and has the ability to complement the yeast *pxa1 pxa2Δ* mutant for β-oxidation of fatty acids [7].

Since only land plants have peroxisomal transporters with the fused heterodimer configuration, but this arrangement is functional in *S. cerevisiae*, we investigated the targeting and functionality of the

Abbreviations: ABC, ATP Binding Cassette; TMD, transmembrane domain; NBD, nucleotide binding domain

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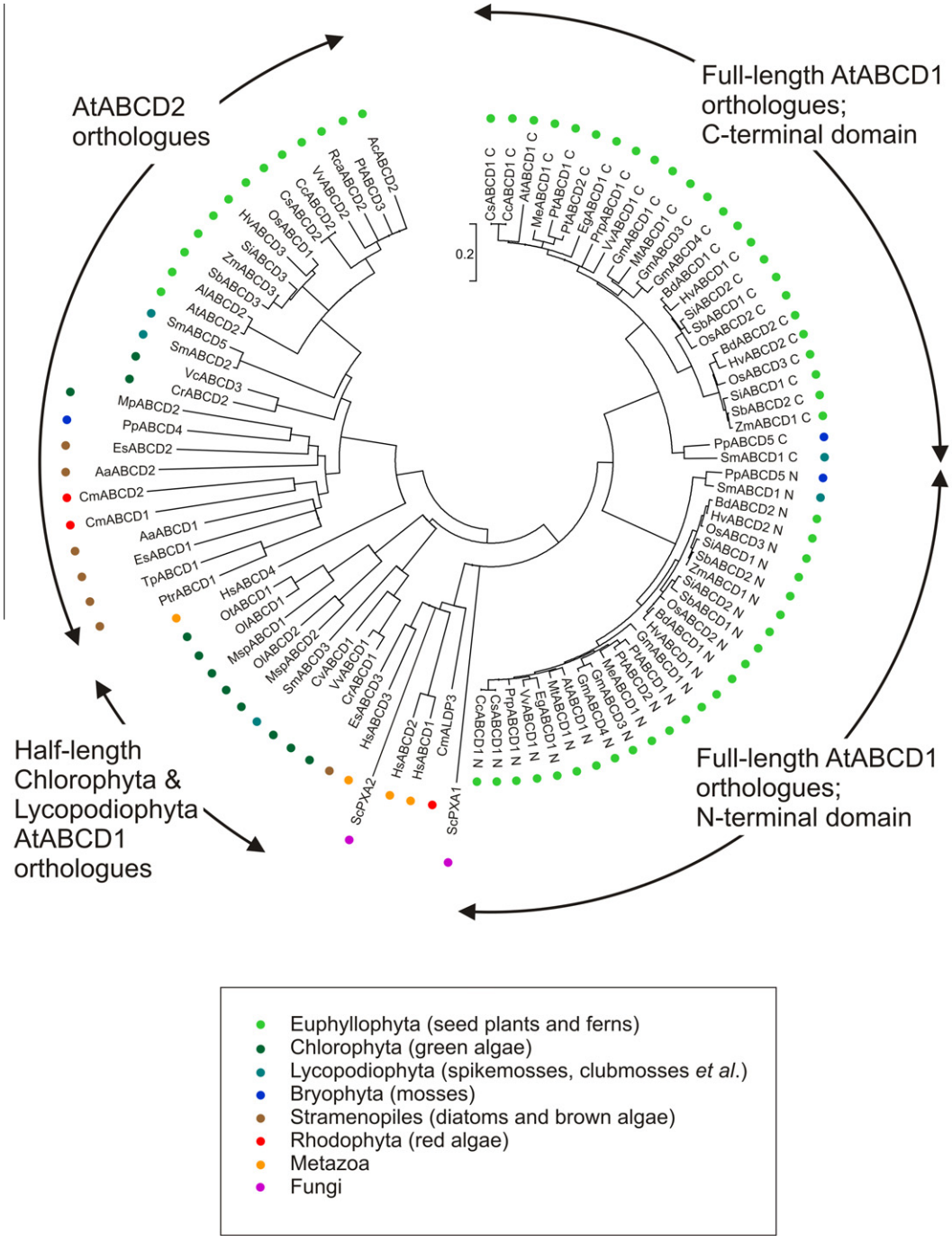

Fig. 1. Phylogenetic analysis of ABC family D transporters. Analysis by the Maximum Likelihood method was performed as described in Supplementary data. Upper and lower case letters at the beginning of protein names indicate abbreviated Latin binomial names for species, as detailed in the species key (*Table* S2). Subfamily members within each species are classified according to [18].

two CTS pseudo half transporters CTS-N and CTS-C from Arabidopsis when expressed in yeast.

2. Materials and methods

2.1. Growth of yeast

Yeast strains were grown as described in [7]. Yeast strains are given in Table 1.

2.2. Pull-down assays

AtPEX19-1 was amplified with primer pair FT119/FT120 (Table S1), restricted with *Bam* HI/*Not* I and cloned in the corresponding sites of pGEX-4T-3 (GE Healthcare). AtPEX19-2 was amplified with primer pair FT121/FT122, restricted with *Sal* I/*Not* I and cloned in the corresponding sites of pGEX-4T-3. Expression and purification of glutathione S-transferase (GST) fusion proteins was carried out as described in [8]. CTS-N and CTS-C were amplified from plasmid H1A6T7 using primer pairs FT187/FT189 and FT190/FT188 respec-

Table 1
Yeast strains used in this study.

BSL1-11B (WT)	*MATa, lys2Δ0, his4Δ519, Ura3Δ0, leu2Δ2*	[19]
BSL1-11B (pxa1Δ)	*MATa, lys2Δ0, his4Δ519, Ura Δ0, leu2Δ2, YPL147w::kanMX4*	[7]
BY4742 (WT)	*Mat a; his3Δ1; leu2Δ00; lys2Δ0; ura3Δ0*	[20]
BY4742 (pex19Δ)	*Mat a; his3Δ1; leu2Δ00; lys2Δ0; ura3Δ0; YDL065c::kanMX4*	[20]
BJ1991	*Matα, leu2, trp1, ura3-251, prb1-112,pex4-3*	[5]
BJ1991 (pxa1,2Δ)	*Matα, leu2, trp1, ura3-251, prb1-112,pex4-3; YPL147w::LEU2;YKL188c::kanMX4*	[5]

tively, restricted with *Nco* I and *Sma* I and cloned into the corresponding sites of pIVEX1.3 (www.5prime.com). In vitro translation was carried out using a wheat germ lysate (5PRIME kit), according to manufacturer's instructions. Aliquots were removed for immunoblot analysis (typically 10–20% input) and equal volumes of the remainder were used for pull-down reactions using GST-AtPEX19-1, GST-AtPEX19-2 or GST alone. Pull-down assays were performed as described in [8], except that proteins were detected by immunoblotting with an anti-penta-His antibody (Qiagen).

2.3. Plasmids for yeast expression

The ORF of AtPEX19-1 was amplified by PCR from plasmid pHD-1PEX19-1 [9] using primer pair AtPEX19F/AtPEX19R (Table S1) and cloned into pRS415-met25 as a *Bam* HI-*Eco* RI fragment. AtPEX19-1 and ScPex19 were amplified from pHD-1PEX19_1 and pKat61 [10] with primer pairs FT163/FT137 and FT160/161, respectively and restricted with *Bam* HI/*Sal* I. AtPEX19-2 was amplified from cDNA clone U13401 (Arabidopsis Biological Resource Center, Ohio) with primer pair FT138/FT139, and restricted with *Bgl* II/*Sal* I. The fragments were ligated into the *Bam* HI/*Sal* I sites of both pRS416/GPD and pRS415/met25. Details of primer pairs are given in Table S1. To generate constructs for the expression of pseudo-half transporters, the N-terminal half of *CTS* (encoding amino acids 1–676, designated "CTS-N") and the C-terminal half ("CTS-C", encoding amino acids 677–1337) were amplified from CTS/pRS416-GPD [7] using primer pairs CTSAF/CTSAR and CTSBF/CTSBR, respectively (Table S1). All products were restricted with *Bam* HI and *Eco* RI and ligated into pRS416-GPD [7] and also pRS415/met25. Similarly, for complementation experiments, CTS-N and CTS-C were amplified using primer pairs FT167/FT168 and FT169/FT170, respectively. Products were restricted with *Bam* HI and *Sal* I and ligated into pEL30 and pIJL30.

2.4. Statistical analysis

Data were expressed as mean SI ± 95% CL, where 95% CL was determined according to the formula: 95% CL = mean ± ($t \times$ SEM), where n = number of samples, t = value of the Student t-distribution at $(n-1)$ degrees of freedom and SEM is the standard error of the mean. All the statistical analysis and calculations were done using Origin® 8.0 Data Analysis and Graphing Software statistical package and Windows® Microsoft Excel.

A

			Score
PMP70 major	----ETGY**LV**K**IAV**M**LV**SR----------	82-96	**10110**
CTS-C major	-----GAQ**LLA**V**AC**L**VV**SR----------	759-773	**7691**
CTS-N minor	----GARD**LLA**L-**V**AT----------	105-119	**3903**
Pxa2p	-----IRT**LL**S**L**R**V**AT**L**DGQLVSTLVRAQY	115-160	**13430**
Pxa1p major	----TPKV**YF**K**LLI**RH**L**LQISRSN------	30-50	**9345**
PMP70 minor	----VDKV**FF**SR**LI**-Q**I**LKI----------	59-73*	**7314**
CTS-C minor 1	-----KAA**FV**R**LI**GLS**V**LQ----------	795-809	**5567**
ALDP	-----NRV**FL**QR**L**LW**LL**RLL----------	68-82*	**37663**
Pxa1p minor	-PTVFDKN**FLL**T**A**Q**IF**FLVMRTWLS----	200-225	**6425**
ALDR	----VNAD**FF**KQ**LLE**L**R**KI----------	80-94*	**19872**
CTS-N major	-----SLR**F**RK**IL**TK**II**HS----------	173-187	**7440**
CTS-C minor 2	-----LRH**L**TQR**LA**LG**W**RIR----------	820-834	**4582**

B

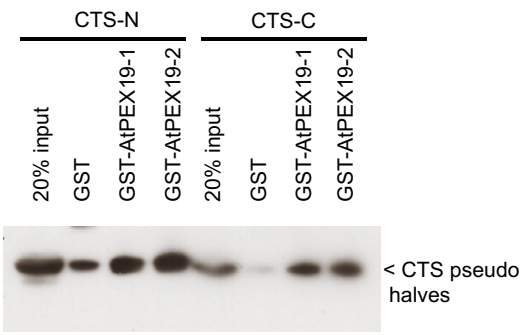

Fig. 2. Role of Pex19 in peroxisomal targeting of CTS. (A) Predicted and experimentally-verified Pex19 binding sites in members of the ABCD family. An algorithm developed for detecting Pex19p binding sites in yeast PMPs was used to detect such binding sites in human, yeast [10] and plant PMPs (H. Rottensteiner, personal communication); scores are given in arbitrary units. An alignment of the Pex19p binding sites in members of the ABC sub family D is presented. Sites marked with an asterisk have been shown to be functional in Pex19 binding assays [12]. (B) Interaction of CTS pseudo-half molecules with Pex19 *in vitro*. AtPEX19-1 and AtPEX19-2 were expressed as GST fusion proteins in *E. coli*. Purified fusion proteins (or GST only, negative control) were immobilised on glutathione beads and incubated with *in vitro* translated histidine-tagged CTS pseudo-half molecules (CTS-N and CTS-C). Interacting proteins were eluted with SDS–PAGE buffer and separated in a 12% acrylamide denaturing gel. Labelled proteins were visualised by immunoblotting with an anti-penta-His antibody. 20% input: 20% of the CTS proteins used in the pull-down experiment. Result representative of three independent experiments.

2.5. Miscellaneous

Peroxisome isolation, carbonate extractions, ATPase assays, immunoblotting and complementation for growth on oleate medium were performed as described in [7] and [9]. Pex19 binding site prediction and homology modelling of CTS are described in [10] and [11], respectively.

3. Results and discussion

3.1. The two halves of CTS contain independent binding sites for both Arabidopsis and Saccharomyces Pex19

Pex19p is a universal and essential factor for the correct localisation and insertion of peroxisome membrane proteins. Although there is debate over the exact role of Pex19p, recognition of peroxisome membrane proteins is central to all the proposed functions. Pex19p binding sites in a number of PMPs are short helical motifs that overlap with the peroxisome targeting signal [10]. A Pex19p binding motif predictor has been developed and used to identify and experimentally verify a Pex19p binding site in human ALDP (HsABCD1) which was functional in both human and *S. cerevisiae* [10,12]. Analysis of CTS with the Pex19p binding site predictor revealed five potential Pex19p binding sites at amino acids 105–119, 173–187, 759–773, 795–809 and 820–834. Based on the scores shown in Fig. 2A, there is a major site in both the N-(CTS-N) and C-(CTS-C) terminal parts of CTS as well as one and two minor sites in these regions, respectively. These sites all share features with those from other ABCD subfamily proteins, especially the predominance of hydrophobic amino acid side chains, the absence of proline and the under representation of negatively charged amino acids.

CTS was expressed as two pseudo-half transporters CTS-N (1–676) and CTS-C (677–1337), dividing the protein just after NBD1, at the beginning of the predicted flexible linker region [7,11], and the ability of each half to interact with different Pex19 isoforms in vitro was examined in a pull-down assay (Fig. 2B). CTS-N and -C were expressed by in vitro transcription and translation, incubated with purified GST-Pex19 fusion proteins and the Pex19 protein recovered by affinity isolation on glutathione agarose beads. GST alone was used as a negative control. Low or very low levels of non-specific binding to the GST control were seen for CTS-N and CTS-C, but much greater amounts (>20% of input) of CTS-N and CTS-C re-isolated with AtPEX19-1, AtPEX19-2 (Fig. 2B) and ScPex19p (Figure S1), indicating that both halves of the protein can interact with Pex19 from both species. The presence of Pex19 binding sites in both halves of the protein likely reflects their distant evolutionary history as separate proteins (see phylogenetic tree in Fig 1). The fact that they have apparently retained functionality over millions of years since the divergence of land plants, suggests that they might be functionally important and that Pex19 interacts with full-length CTS at more than one site, perhaps to maintain solubility prior to insertion in the peroxisomal membrane. Large membrane proteins are difficult for the cell to fold and are often subject to a high rate of misfolding, thus chaperones would be selected for. This has been very well documented for another "full-length" ABC transporter, the cystic fibrosis transmembrane conductance regulator, CFTR, which is subject to multiple quality control mechanisms [13].

3.2. Arabidopsis PEX19 cannot complement the S. cerevisiae pex19Δ mutant for growth on oleate

Since CTS can be recognised by ScPex19p and since correct targeting of human [12] and plant [7] PMPs has been demonstrated in

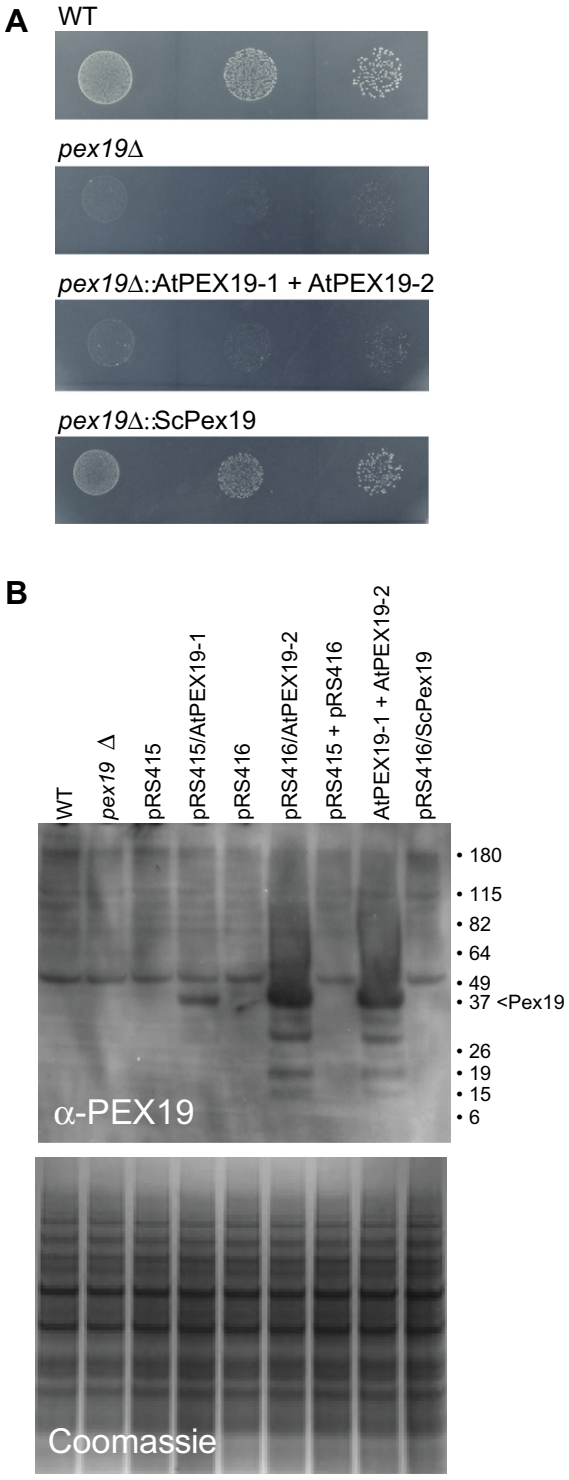

Fig. 3. Arabidopsis PEX19 does not complement the yeast *pex19Δ* mutant for growth on oleate. (A) Growth of wild type (WT) and *pex19Δ* cells on solid oleate medium. *pex19Δ* cells were transformed with AtPEX19-1/pRS416, AtPEX19-2/pRS415, both Arabidopsis Pex19 isoforms, ScPex19/pRS416 or vectors lacking insert. Complementation was not observed when *pex19Δ* cells were transformed with either AtPEX19-1 or AtPEX19-2 alone (not shown). (B) Extracts from cells in (A) separated on a 4–12% SDS–PAGE gradient gel and immunoblotted with a polyclonal antiserum raised against AtPEX19-1 but which also recognises AtPEX19-2 ([9]; upper panel) or stained with Coomassie Brilliant Blue (lower panel). The mobilities of marker proteins of known mass (kDa) are indicated on the right hand side of the immunoblot. The different levels of apparent expression likely reflect the strength of the promoters in pRS416 and pRS415.

S. cerevisiae, we tested whether Arabidopsis PEX19 could complement the *S. cerevisiae pex19Δ* mutant for growth on oleate. The two PEX19 isoforms of Arabidopsis, PEX19-1 and PEX19-2, are 79% identical at the amino acid level but share only ca. 25% identity to ScPex19p [9]. RNAi down regulation of these two Pex19 isoforms in Arabidopsis suggested they are not completely functionally redundant [14]. We expressed AtPEX19-1 and AtPEX19-2 independently and together in the *S. cerevisiae pex19Δ* mutant (Fig. 3A). Neither protein alone or in combination was able to rescue the oleate non-utilisation (*onu*) phenotype of the *pex19Δ* mutant, despite both proteins being expressed at detectable levels (Fig. 3B). This may be because the ability to complement the *onu* phenotype is a very stringent test of conservation of function and requires not only recognition of the targeting signals of all PMPs necessary (directly or indirectly) for oleate metabolism, but also rescue of all Pex19 functions, including the capacity to be modified by the yeast farnesyl transferase and to interact productively with yeast Pex3p.

3.3. CTS-N and CTS-C target independently to peroxisomes and are correctly integrated into peroxisome membranes

CTS-N and CTS-C were expressed separately in yeast and oleate-grown cultures were subjected to fractionation on Optiprep™ gradients to prepare peroxisome- and mitochondria-enriched fractions, which were subsequently analysed by SDS PAGE and immunoblotting (Fig. 4). Good separation between the organellar fractions was demonstrated by the enrichment of thiolase (peroxisomal marker) and porin (mitochondrial marker) in the appropriate fractions (Fig. 4A, lower two panels). Both CTS-N and CTS-C were almost exclusively detected in the peroxisome fraction (Fig. 4A upper panel), with a small amount being detected in the mitochondrial fraction. When the peroxisome membrane is limiting in yeast cells (for example on lactate medium or in the *pex19Δ* background), CTS mistargeted and was detected in the mitochondrial fraction (data not shown). CTS-N and CTS-C were found to be more stable than the full length CTS, which is subject to extensive proteolysis within the linker region joining the two pseudo halves [7]. This is in contrast to Pxa1p, which is unstable in the *pxa2Δ* background, although Pxa2p is stable in the absence of Pxa1p [15]. Similarly, the mammalian ER-located ABC half-transporter, TAP2, is unstable in the absence of its partner TAP1 [16]. Both CTS-N and CTS-C were not extractable from the membranes by alkaline sodium carbonate treatment, consistent with the fact that they contain multiple transmembrane helices and are presumably integrated into the membrane (Fig. 4B). These results demonstrate that each pseudo half transporter contains all the necessary information to be recognised and inserted into peroxisome membranes by the heterologous host cell machinery.

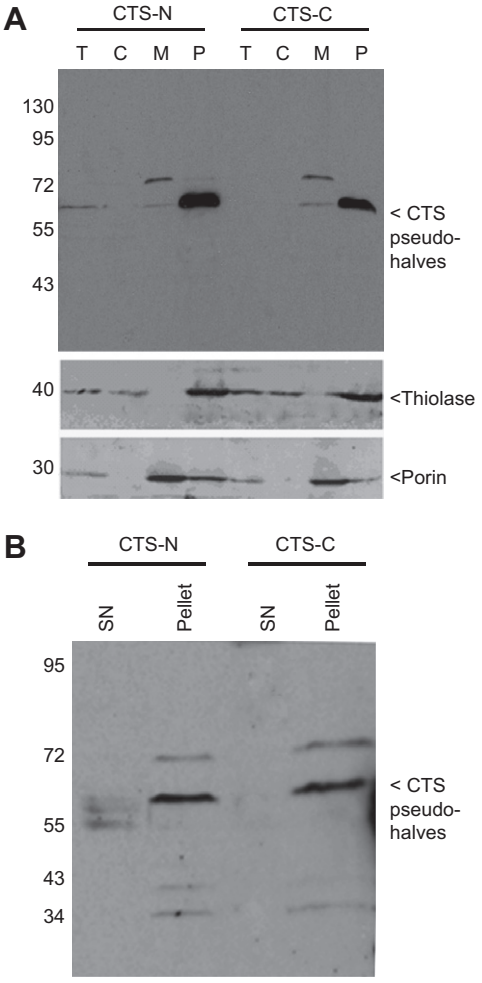

Fig. 4. CTS N- and C-terminal pseudo-half molecules target to peroxisomes independently and are integrated into the peroxisome membrane. (A) BLS1-11B cells transformed with the CTS-N/pRS416-GPD or CTS-C/pRS416-GPD were grown on WOYD medium and transferred to the oleate medium for peroxisome induction. A light mitochondrial fraction was prepared and separated on an iodixanol density gradient. The fractions corresponding to the peroxisomes (P) and mitochondria (M) were separated by SDS–PAGE in a 7.5% gel (30 μg of protein/lane) and immunoblotted with antibodies raised against CTS, thiolase (peroxisomal marker), and porin (mitochondrial marker) [7]. The fractions corresponding to the total cell lysate (T) and cytosol (C) were also collected and treated as above. The mobilities of marker proteins of known mass (kDa) are indicated on the left hand side of the figure. The upper band represents unrelated cross reaction with a mitochondrial protein. (B) Organelles (“light mitochondrial fraction”) were isolated from oleate-grown BLS1-11B cells transformed with the CTS-N/pRS416-GPD or CTS-C/pRS416-GPD and were lysed in a hypotonic buffer in the presence of protease inhibitors. Membranes were separated from soluble proteins by centrifugation at 100,000*g* and then extracted with alkaline sodium carbonate buffer, pH 11.5, followed by re-centrifugation at 100,000*g*. Pellet and supernatant (SN) fractions were separated by SDS–PAGE (30 μg per lane) followed by immunoblotting with antibodies raised against CTS. The mobilities of marker proteins of known mass (kDa) are indicated on the left hand side of the figure.

3.4. Co-expression of CTS-N and CTS-C is required for function

We investigated whether CTS-N and CTS-C are functional when expressed in yeast. Neither protein complemented the *onu* phenotype of the *pxa1,2Δ* mutant when expressed alone, but when expressed together permitted growth on oleate comparable to that obtained with full-length CTS (Fig. 5A). We then tested whether the pseudo-halves could form homodimers or heterodimers with ATPase activity. ATPase activity was measured in peroxisomes isolated from strains expressing full length CTS, CTS-N and CTS-C separately, CTS-N and CTS-C co-expressed, and empty vector transformed cells. Peroxisomes from cells co-expressing CTS-N and CTS-C had CTS-specific basal ATPase activity (as measured in [7]) which was almost as high as that of peroxisomes containing the full length CTS. In contrast, peroxisomes from cells where CTS-N and CTS-C were expressed alone had no more ATPase activity than control cells. Thus CTS-N and CTS-C can assemble to form an active heterodimer. CTS-N and CTS-C alone are either unable to homodimerise, or form an inactive homodimer.

As NBDs dimerise during the transport cycle, the two ATP binding sites are composites, comprising residues from both NBDs. Each ATP site is made up of the Walker A, Walker B and Switch motif (H-loop) from one NBD and the signature motif and D loop from the other NBD. NBD1 of CTS shows conservation of all functionally important residues in the Walker A, Walker B and Switch or ‘H’ loop and mutagenesis shows that Walker A and B residues of NBD1 are essential for function in vivo [11]. In contrast, NBD2

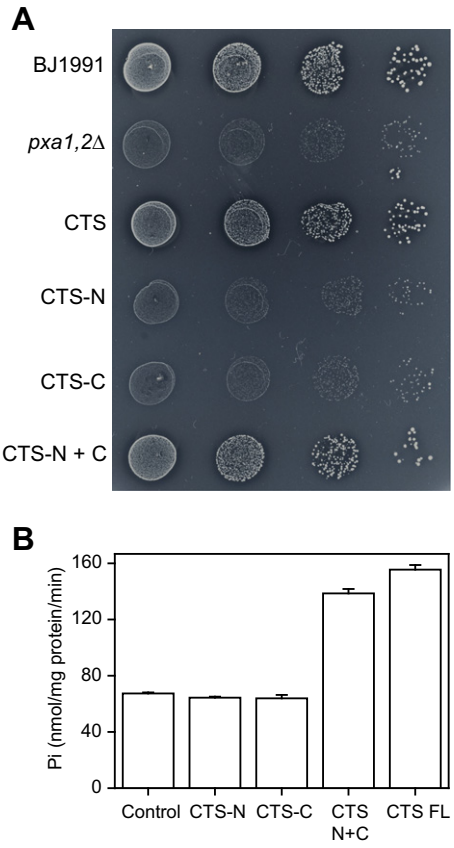

Fig. 5. Functional analysis of CTS pseudo-half molecules. (A) Growth of wild-type and mutant cells transformed with the indicated constructs on solid oleate medium. (B) ATPase activity in peroxisomes isolated from oleate-grown wild-type BSL1-11B yeast, transformed with CTS-N/pRS416-GPD, CTS-C/pRS415-met25, singly and in combination and CTS/pRS416-GPD (CTS FL) or vector lacking an insert (control). All samples were analysed in triplicate, and the experiment was repeated three times. The data were used to compute the means and the standard error of the mean (SEM). Data are expressed as mean SI ± 95% CL, where 95% CL was determined according to the formula: 95% CL = mean ± ($t \times$ SEM), where n = number of samples, t = value of the Student t-distribution at ($n - 1$) degrees of freedom and SEM is the standard error of the mean.

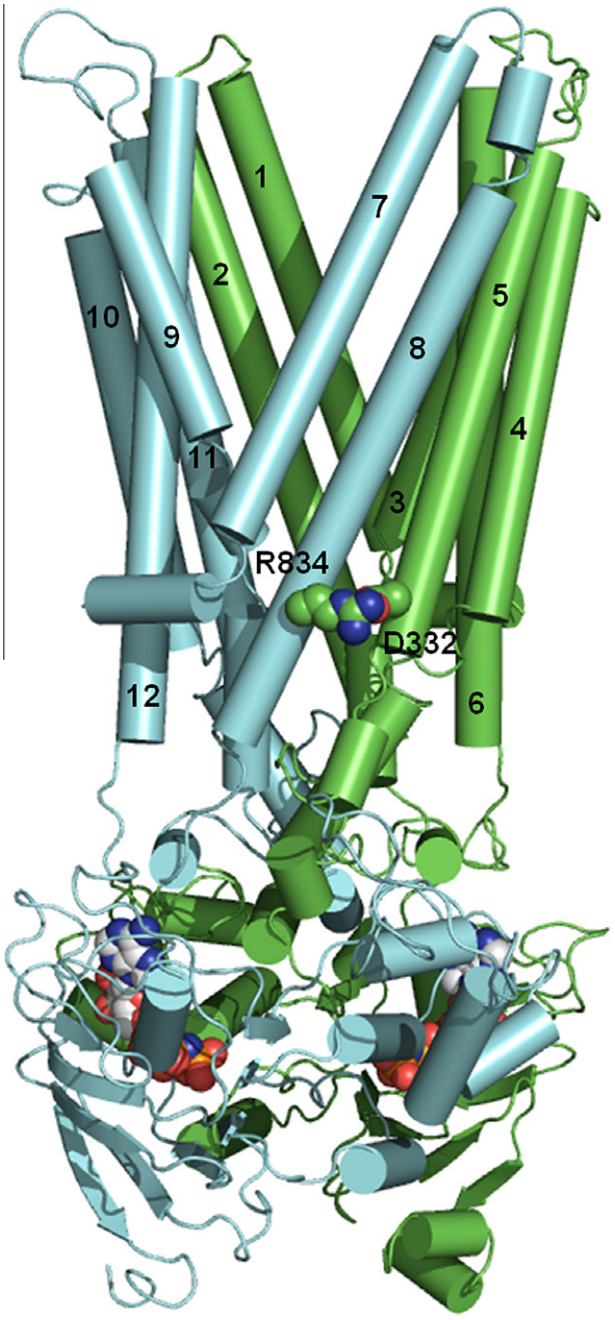

Fig. 6. Homology model of CTS showing domain swapping of transmembrane helices. Modified from [11]. The N- and C-terminal halves of the CTS molecule are coloured in green and blue, respectively. Residues R834 and D332, predicted to form a salt bridge in the interface between the halves, are shown in space-filling representation, as are bound AMP-PNP molecules within the NBDs.

has Q in place of H in the Switch loop. This histidine plays a critical role in contacting the γ-phosphate of the bound ATP. Thus CTS will have one consensus and one degenerate ATP binding site. This asymmetry is quite common in eukaryotic ABC transporters [17 and references therein]; indeed Pxa1p and TAP1 also contain the same H to Q substitution. In the light of this, CTS-C might be expected to be non-functional when expressed in the absence of CTS-N. However, CTS-N contains consensus residues which contribute to the ATP binding site and might be predicted to form a catalytically active homodimer when expressed alone. The fact that CTS-N expressed alone does not exhibit ATPase activity suggests either, that it is not able to homodimerise, and/or that a "degenerate" ATP binding site is somehow important for the ATPase catalytic cycle of CTS. Alternatively, the lack of functionality of CTS pseudo half molecules may be related to TMD structure. A model of CTS based on the structure of the homodimeric ABC transporter Sav1866 predicts "domain-swapping", such that TMD1 contacts NBD2 and vice versa [11]. Moreover, in the model, α-helices from the two distinct halves of the protein are shared between the two TMDs, such that one is composed of TM helices 3–8 and the other of TM helices 1, 2, and 9–12 (Fig. 6). Within the TMDs, the interface between the two halves of the protein involves interactions between TM2 and 11 and between TM8 and 5. In the homodimeric molecule Sav1866 the equivalent interactions, between TM2 of one subunit and TM5 of the other, are necessarily identical whereas this is not the case in native CTS. For example, a salt bridge is predicted between R834 (TM8) and D332 (TM5), while the equivalent positions in the other TMD are occupied by I180 (TM2) and K986 (TM11). In the CTS-C homodimer, the corresponding interface would contain R834 and K986, likely resulting in electrostatic repulsion, while in the CTS-N homodimer, the interface would contain I180 and D332, precluding salt bridge formation or the likely hydrophobic interaction between the isoleucine side chain and the aliphatic portion of the lysine side chain. Similar differences in size and/or charge are observed between a number of other conserved interfacial residues at equivalent posi-

tions elsewhere in the two halves of the molecule. These would likely interfere with the association between, and/or activity of, the CTS homodimers. Conservation of a number of interfacial residues is indicative of their probable collective importance in maintaining the interaction between the helices. In conclusion, while there is no apparent requirement for the two halves of CTS to be covalently joined to produce a functional molecule, both halves are indispensable for biological activity.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.05.065.

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