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# The *Saccharomyces cerevisiae* ABC subfamily D transporter Pxa1/Pxa2p co-imports CoASH into the peroxisome

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**ATP-binding cassette (ABC) subfamily D transporters are important for the uptake of fatty acids and other beta-oxidation substrates into peroxisomes. Genetic and biochemical evidence indicates that the transporters accept fatty acyl-coenzyme A that is cleaved during the transport cycle and then re-esterified in the peroxisomal lumen. However, it is not known whether free coenzyme A (CoA) is released inside or outside the peroxisome. Here we have used *Saccharomyces cerevisiae* and isolated peroxisomes to demonstrate that free CoA is released in the peroxisomal lumen. Thus, ABC subfamily D transporter provide an import pathway for free CoA that controls peroxisomal CoA homeostasis and tunes metabolism according to the cell's demands.**

**Keywords:** ABC transporter; acyl-CoA; coenzyme A; coenzyme homeostasis; peroxisome; thioesterase

Peroxisomes are essential organelles in eukaryotic cells and are involved in different metabolic processes, including fatty acid β-oxidation. These metabolic functions require that many substrates, products and cofactors be able to cross the peroxisomal membrane. ATP-binding cassette (ABC) transporters belonging to subfamily D mediate import of fatty acids (FA), whereas mitochondrial carrier family (MCF) transporters have been associated with cofactor import [1–3].

Two different models accounting for fatty acid transport by peroxisomal ABC subfamily D transporter (ABCD) proteins have been proposed [4]. One model, based on data from human fibroblasts, suggests that acyl-coenzyme A (acyl-CoA) binds to the ABCD transporter and is transported across the peroxisomal membrane [5]. In the other model, which builds on observations made in yeast and plants, acyl-CoAs are bound by the ABCD transporters at the cytosolic side of the peroxisomal membrane, hydrolysed during transport and delivered as an unesterified fatty acid to the luminal side of the peroxisomal membrane. A peroxisomal synthetase, which is CoA- and ATP-dependent, then re-activates the unesterified fatty acid by converting it into acyl-CoA in the peroxisomal matrix [6–8].

Several observations provide evidence for the second model. Firstly, peroxisomal acyl-CoA synthetase activity associated with the matrix side of the peroxisomal

**Abbreviations**
3-HAD, 3-hydroxyacyl-CoA dehydrogenase; ABC, ATP-binding cassette; ABCD, ABC subfamily D transporter; ACOT, acyl-CoA thioesterase; acyl-CoA, acyl-coenzyme A; CoA, coenzyme A; CoASH, free coenzyme A; unit, μmol·min$^{-1}$.

membrane is required for ABCD-dependent transport in plants and yeast [6–8]. Secondly, $^{18}$O-labelling of β-oxidation intermediates was used to demonstrate that acyl-CoA hydrolysis is an obligatory step prior to oleate β-oxidation in yeast, although the source of the acyl-CoA thioesterase (ACOT) activity involved was not identified [7]. Finally, expression of the *Arabidopsis thaliana* ABCD transporter, AtCTS in insect cells has shown that ACOT activity is an intrinsic property of the transporter itself and is not catalysed by a separate peroxisomal enzyme [8]. This result was confirmed subsequently using *Saccharomyces cerevisiae* peroxisomes [9], and ACOT activity was also demonstrated for purified, recombinant human ABCD proteins expressed in *Pichia pastoris* [10].

The physiological significance of this mechanism is currently unclear, given that the fate of the cleaved CoA moiety is unknown. Cleaving the acyl-CoA ester to produce a polar CoA moiety and a relatively hydrophobic free fatty acid may provide a solution to the biophysical problem of transporting an amphipathic molecule across the peroxisomal membrane [11]. Additionally, cleavage may contribute to regulation of the cytosolic and peroxisomal CoA pools, enabling the peroxisome to prioritise β-oxidation of different substrates and optimise flux. To date, a comprehensive understanding of peroxisomal cofactor homeostasis is lacking, although several MCF transporters that accept free coenzyme A (CoASH), ATP or $NAD^+$ have been identified. For example, yeast and plant peroxisomal ATP carriers have been characterised *in vivo* and *in vitro*, but no functionally equivalent carrier has yet been identified in humans [12–15]. A putative peroxisomal CoA carrier has been described in humans, although its *in vivo* substrates have not been confirmed [3,16,17]. Similarly, whilst an Arabidopsis peroxisomal carrier has been identified that accepts CoASH and derivatives *in vitro* [18], kinetic considerations and *in vivo* experiments demonstrate that the physiological role of this transport protein is to exchange $NAD^+$ for AMP [19,20]. The potential contribution of ABCD transporters to peroxisomal CoA homeostasis depends on their transport mechanism. If acyl-CoAs are transported intact, then the ABC transporters mediate net import of CoA. This is also the case if cleaved CoA is released inside the peroxisome, but in a model where cleaved CoA is released in the cytosol, then the peroxisome would be entirely dependent on an alternative route for CoA import. To obtain more insight into the regulation of peroxisomal CoA homeostasis, we used *S. cerevisiae* to demonstrate that a CoASH molecule from acyl-CoA is transported *via* the ABCD transporter and released in the peroxisomal lumen. Thus, ABCD transporters provide an import pathway for free CoA.

## Materials and methods

### Yeast strains and culture conditions

*Saccharomyces cerevisiae* mutants were constructed in the strain BJ1991 (MATα, *pep4-3*, *prbl-1122*, *ura3-52*, *leu2*, *trp1*) [21,22]. Cells were grown in medium containing 5 g·L$^{-1}$ D-glucose, 6.7 g·L$^{-1}$ and yeast nitrogen base without amino acids (Difco, Thermo Fisher Scientific, Waltham, MA, USA, https://www.fishersci.com). Supplements (leucine, uracil, or tryptophan; 30, 20 and 20 mg·L$^{-1}$, respectively) were added where required. To induce peroxisome proliferation, cells were grown for at least 24 h in glucose medium and then transferred to YPO medium (1.07 g·L$^{-1}$ oleate, 2.16 g·L$^{-1}$ Tween-80, 5 g·L$^{-1}$ peptone, 3 g·L$^{-1}$ yeast extract, 25 mM potassium phosphate buffer, pH 6).

### Peroxisome isolation

The method described in [9] was used with slight modifications, as described below. One litre of *S. cerevisiae* was grown overnight in YPO medium to $OD_{600}$ = 1.2 ($1OD_{600}$ = 1.48 × $10^7$ cells). Cells were harvested, washed and incubated in 7.5 mL buffer A (0.1 M Tris/sulfate pH 9.4) plus 10 mM DTT for 20 min at 28 °C. Cells were then washed in 20 mL buffer B (1.2 M Sorbitol, 50 mM KPi pH 7.5, 1 mM EDTA) and incubated in 1.5 mL buffer B containing 0.75 mg·mL$^{-1}$ Zymolyase 20T. After 15–20 min, spheroplasts were collected by centrifugation (2310 ***g*** 5 min) and washed with buffer C (1.2 M sorbitol, 25 mM MES pH 6.0, 1 mM KCl, 1 mM EDTA). Spheroplasts were subjected to three further rounds of resuspension in buffer D (0.6 M sorbitol, 25 mM MES pH 6.0, 1 mM KCl, 1 mM EDTA), and supernatants were combined. The combined supernatants were centrifuged at 4110 ***g*** for 10 min at 4 °C to remove cell debris, nuclei and large organelles. Five hundred microlitre of homogenate fraction was used for the β-oxidation assay, and the remainder was used for cell fractionation. Homogenates (14.5 mL) were centrifuged at 27 000 g at 4 °C in an SS34 rotor (Sorvall) for 30 min. Subsequently, the pellets (MP) containing peroxisomes and mitochondria were collected and gently suspended in 1 mL of buffer D and loaded on top of a Nycodenz step gradient (150–200–250–300–350 g·L$^{-1}$) in 10 mM MES-KOH, pH 6.0, 1 mM KCl, 1 mM EDTA and 85 g·L$^{-1}$ sucrose. Centrifugation was carried out in a vertical rotor at 43 000 ***g*** 4 °C for 150 min and fractionated in 10 fractions (fraction 1–6 1 mL; 7–10 1.5 mL) (Fig. S1). 0.83 mL of fractions 2, 3 and 4 were combined and diluted 1 : 1 with buffer D. After homogenisation, 1.5 mL was centrifuged at 1 072 000 g in optima™ Max-xp ultracentrifuge at 4 °C for 60 min. The supernatant was removed, and the

peroxisomal pellet (P) was directly used for the transport assays after resuspension in buffer D or stored at –80 °C for further analysis. Peroxisomes were isolated in duplicate from each cell line for enzyme assays, and the entire experiment was independently replicated giving a total of three peroxisome pellets per assay per cell line.

### 3-hydroxyacyl-CoA dehydrogenase (3-HAD) assay

The activity of the peroxisomal marker 3-HAD was used to quantify the amount of peroxisomes during cell fractionation and peroxisomal isolation. Activity was measured by monitoring the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm, using a Cobas Fara centrifugal analyser [23]. Total 3-HAD activity was comparable in all strains.

### Fumarase assay

Fumarase activity was determined as described in Ref. [7].

### ATPase assay in isolated yeast peroxisomes

ATPase activity was determined by an enzyme-linked spectrophotometric assay from [9] with some slight modifications, as described below. Assay medium (50 mM KPi pH 8.0, 50 mM HEPES pH 8.0, 0.3 mM NADH, 4 mM phosphoenolpyruvate, 7.5 units pyruvate kinase, 8.8 units lactate dehydrogenase, 1 mM DTT) was pre-incubated with 20 µL isolated peroxisomes (10 units per mL 3HAD) for 5 min at 28 °C. Reactions were started with 25 µL of start solution (70 mM $MgSO_4$, 50 mM ATP, 0.2 mM BSA plus or minus 17 µM oleoyl-CoA). Final volume of the assay was 250 µL. The reaction was followed for 30 min on Cobas Fara centrifugal analyser at 340 nm.

### [1-$^{14}$C]Myristate β-oxidation in intact cells

The method described in Ref. [9] was used. Briefly, cells were grown overnight in YPO medium to induce peroxisome proliferation. Cells were washed and resuspended in NaCl 9 g·L$^{-1}$ at a cell density of $OD_{600}$ = 2 ($1OD_{600}$ = 1.48 × $10^7$ cells). Incubations were performed by adding 20 µL of MES buffer (0.5 M; pH = 6), 20 µL of cells $OD_{600}$ = 2 ($1OD_{600}$ = 1.48 × $10^7$ cells), 140 µL NaCl (9 g·L$^{-1}$) and 20 µL of 100 µM [1-$^{14}$C] myristate (200 000 dpm; ARC 0198) as substrate. The reaction was allowed to proceed for 1 h at 28 °C, at which point, the reaction was terminated by the addition of 50 µL of perchloric acid (final concentration: 0.52 M), and radiolabelled [$^{14}$C]-$CO_2$ was trapped in a tube containing 500 µL 2 M NaOH. Radiolabelled $CO_2$ and acid-soluble β-oxidation products produced were used to quantify the rate of fatty acid oxidation *via* a liquid scintillation counter.

### [1-$^{14}$C]Myristoyl-CoA β-oxidation in lysates

The reaction mixture contained 20 µL homogenate (0.2 mg·mL$^{-1}$ protein), 10 µM [1-$^{14}$C]Myristoyl-CoA (Moravek Biochemicals: MC1467, Brea, CA, USA, https://www.moravek.com), 1 mM NAD, 50 mM Tris pH 8.5, 10 mM ATP, 10 mM $MgCl_2$, 50 µM FAD and 0.5 g·L$^{-1}$ Triton X-100 in a total volume of 200 µL. Reactions were allowed to proceed for 10 or 20 min at 28 °C, followed by termination by adding 50 µL perchloric acid (2.6 M). The [1-$^{14}$C]-labelled β-oxidation products were subsequently collected after extraction with chloroform/methanol/heptane as described before [24]. The radiolabelled acid-soluble counts were used to quantify the rate of fatty acid oxidation by liquid scintillation counting.

### [1-$^{14}$C]Myristoyl-CoA β-oxidation in isolated peroxisomes

We used the method described in Ref. [5], but without addition of a cytosolic fraction lacking membranous structures. The reaction medium contained 5 µM [1-$^{14}$C]Myristoyl-CoA (100 000 dpm), 30 mM KCl, 1 mM NAD, 20 mM HEPES pH 7.4, 175 µM FAD, 2.5 mM carnitine, 0.16 mM CoASH, 0.5 mM malate, 0.2 mM EDTA, 1 mM DTT, 250 mM sucrose, with or without 10 mM ATP and with or without 10 mM $MgCl_2$. Reactions were started by the addition of 20 µL isolated peroxisomes (20 units per mL of 3HAD) in a total volume of 200 µL and allowed to proceed for 18 min at 28 °C and terminated by adding 50 µL 2.6 M perchloric acid. The [1-$^{14}$C]-labelled β-oxidation products were collected after extraction with chloroform/methanol/heptane as described in Ref. [25]. The radiolabelled acid-soluble counts were used to quantify the rate of fatty acid β-oxidation in isolated peroxisomes by liquid scintillation counting.

### Preparation of myristoyl-[$^3$H]CoA

The reaction mixture contained 20 µL of 2.5 U·mL$^{-1}$ synthetase activity (suspended in 100 g·L$^{-1}$ glycerol) (Sigma, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, https://www.sigmaaldrich.com; from *Pseudomonas*), 200 µM myristate (C14:0), 100 mM Tris pH 8.5, 5 mM ATP, 5 mM $MgCl_2$, 0.5 mM DTT, 1 g·L$^{-1}$ Triton X-100 and 1 µM tritiated coenzyme A (CoA) ([4'-phosphopantetheine-$^3$H]; Hartmann Analytic, Braunscheig, Germany, https://www.hartmann-analytic.de; Specific activity: 5–20 Ci·mmol$^{-1}$) in a total volume of 1 mL. After 50-min incubation at 28 °C, the reaction was terminated by 750 µL Doles reagent (isopropanol:heptane : 2N $H_2SO_4$ 40 : 10 : 1). Myristoyl-[$^3$H] CoA was separated from myristate by a method described in Ref. [26]. The myristoyl-[$^3$H] fraction was further purified using a C18 reverse phase column. The column was activated with 5 mL of methanol (fraction 1) and 1 mL of

sample loaded (fraction 2). The column was eluted sequentially with 4 mL of Tris pH 7.4 (fraction 3), 4 mL of 30% (v/v) methanol (fraction 4), 4 mL of 50 % (v/v) acetonitrile (fraction 5), 4 mL of 90% (v/v) acetonitrile (fraction 6) and 4 mL acetonitrile (fraction 7). The separation of free $^{3}$H-CoA (Hartmann Analytic; Specific activity: 5–20 Ci·mmol$^{-1}$), [1-$^{14}$C]-acetyl-CoA (Amersham; CFA729) and [1-$^{14}$C]-myristoyl-CoA standards is shown in Fig. S2. Fractions 5 and 6 were combined (8 mL), evaporated and then redissolved in 500 µL of 100 mM Tris pH 8.5, 10 mg·mL$^{-1}$ α-cyclodextrin. The myristoyl-[$^{3}$H]CoA stock solution was 4000 dpm µL$^{-1}$.

### Myristoyl-[$^{3}$H]CoA import into isolated peroxisomes

The reaction contained 40 000 dpm Myristoyl-[$^{3}$H]CoA (5 µM), 30 mM KCl, 1 mM NAD, 20 mM HEPES pH 7.4, 175 µM FAD, 2.5 mM carnitine, 0.16 mM CoASH, 0.5 mM malate, 0.2 mM EDTA, 1 mM DTT, 250 mM sucrose, with or without 10 mM ATP and with or without 10 mM $MgCl_2$. Reactions were started by the addition of 20 µL isolated peroxisomes (20 units per mL 3HAD) in a total volume of 200 µL, allowed to proceed for 18 min at 28 °C and terminated by separation on a Sephadex 75 column. The column was pre-equilibrated with 0.25 M sucrose, 10 mM KCl and 10 mM HEPES pH 7.4. As shown in Fig. S3, 10 fractions were collected and radiolabelled myristoyl-[$^{3}$H]CoA and [$^{3}$H]CoA were measured by liquid scintillation counting. The peroxisomal fractions (4 and 5) were added to a C18 reverse phase column and the radiolabelled products [$^{3}$H]CoA, acetyl-[$^{3}$H]CoA and myristoyl-[$^{3}$H]CoA separated by stepwise elution, as described above (Fig. S2).

## Results

### Selection of experimental system: myristoyl-CoA is a substrate of Pxa1/Pxa2p

Yeast is an excellent model system to study β-oxidation because this pathway is restricted to the peroxisome, unlike in mammalian cells, where β-oxidation is initiated in the peroxisome and completed in the mitochondria [1]. Yeast peroxisomes contain two fatty acid import pathways: an ABCD-dependent pathway for acyl-CoAs and an ABCD-independent free fatty acid import pathway that requires the peroxisomal acyl-CoA synthetase, Faa2p [21,22]. These pathways differ in substrate specificity. The heterodimeric peroxisomal ABCD transporter Pxa1/Pxa2p accepts medium, long and very long-chain acyl-CoA esters as substrates [21,22,27–29]. However, because yeast lacks microsomal or cytosolic acyl-CoA synthetases with specificity for octanoate (C8:0) and laurate (C12:0), these medium-chain FA are exclusively imported into the peroxisome *via* the free fatty acid import pathway after which they are activated by the peroxisomal acyl-CoA synthetase Faa2p [22]. As the aqueous solubility of acyl-CoA species decreases with increasing acyl chain length [30,31], we first set out to identify the shortest fatty acid that can be activated outside the peroxisome and thus become a substrate for Pxa1/Pxa2p transporter *in vivo*. As shown in Fig. 1A, the β-oxidation of myristate (C14:0) was strongly impaired in intact *faa2/pxa1/pxa2Δ* cells, which lack both fatty acid import pathways, and, as a control, in *fox1Δ* cells, which lack acyl-CoA oxidase, the enzyme that catalyses the first committed step of β-oxidation. *faa2Δ* and *pxa1/pxa2Δ* cells exhibited 21 % and 71 % of wild-type β-oxidation activity, respectively. The residual activity in *faa2Δ* cells is likely due to a peroxisomal

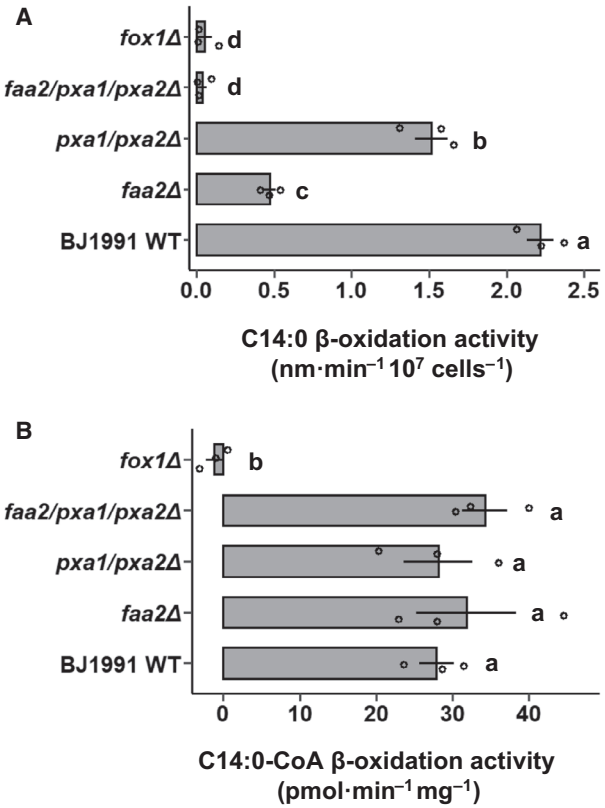


**Fig. 1.** Myristate is imported into yeast peroxisomes by two pathways. (A) [1-$^{14}$C]Myristate (C14:0) β-oxidation in intact cells of mutant and wild-type yeast; (B) [1-$^{14}$C]Myristoyl-CoA (C14:0-CoA) β-oxidation in detergent-treated cell lysates. β-oxidation activity is expressed as the sum of radiolabeled $CO_2$and water-soluble β-oxidation products produced. Values are the means ± SE of three independent experiments. Letters indicate statistically significant differences (compact letter display format; Tukey's HSD test; $P \leq 0.01$).

subpopulation of the acyl-CoA synthetase, Fat1p, that is functionally coupled to Pxa1/Pxa2p, as demonstrated in Ref. [7]. Importantly, myristoyl-CoA β-oxidation was normal in *pxa1/pxa2Δ*, *faa2Δ* and *faa2/pxa1/pxa2Δ* cell-free lysates in which the membrane barriers of the different intracellular organelles were disrupted, and cofactors are present in excess (Fig. 1B). This indicates that the impairment of myristate β-oxidation in intact cells is caused solely by the absence of Pxa1/Pxa2p, Faa2p or both and not by impaired activity of the β-oxidation enzymes.

## Isolated peroxisomes exhibit substrate-stimulated Pxa1/Pxa2p-dependent ATPase activity and ATP-dependent acyl-CoA β-oxidation activity

We next set up a fatty acid transport assay using peroxisomes isolated from wild-type, *pxa1/pxa2Δ*, *faa2Δ* and *faa2/pxa1/pxa2Δ* cells (Fig. S1). Prior to carrying out transport assays, it was important to test the functionality of Pxa1/Pxa2p in the different preparations. Substrate stimulation of basal ATPase activity is a hallmark of many ABC transporters, including those belonging to subfamily D [32], and provides a convenient assay for this purpose. In agreement with previous studies [32], peroxisomes from *pxa1/pxa2Δ* and *faa2/pxa1/pxa2Δ* cells exhibited a low level of background ATPase activity that was not influenced by acyl-CoA (Fig. 2). This is probably due to the presence of other ATPases in the peroxisome membrane, such as AAA-ATPases [33,34]. Importantly, however, ATPase activity was higher in wild-type and *faa2Δ* peroxisomes and was stimulated ~ 1.5-fold by the addition of acyl-CoA (Fig. 2). This suggests that the isolated peroxisomes from wild-type cells and *faa2Δ* mutants contain substrate-stimulated Pxa1/Pxa2p transporter activity and can be used for myristoyl-CoA β-oxidation measurements *in vitro*.

To study fatty acyl-CoA uptake, [1-$^{14}$C]-myristoyl-CoA β-oxidation activity was measured in isolated peroxisomes from wild-type and mutant cells. Addition of ATP stimulated the β-oxidation of [1-$^{14}$C]-myristoyl-CoA by wild-type peroxisomes (Fig. 3), consistent with the requirement of ATP hydrolysis for transport and possibly also for peroxisomal acyl-CoA synthetase activity. *pxa1/pxa2Δ* and *faa2/pxa1/pxa2Δ* peroxisomes had a low level of β-oxidation activity which most likely represents background due to leaky peroxisomes, since Pxa1/Pxa2p is absent in these assays. As ATP did not enhance β-oxidation in *pxa1/2Δ* peroxisomes (in contrast to wild-type peroxisomes), we concluded that a significant proportion of the peroxisomes is intact. This result also indicates that myristoyl-CoA is stable in the assay medium, as previously reported [35], and that there is no significant hydrolysis of myristoyl-CoA thereby releasing free myristate to act as a substrate for the Faa2p pathway. ATP-stimulated β-oxidation of myristoyl-CoA was also not observed in peroxisomes isolated from *faa2Δ* cells. This is consistent with the requirement of a peroxisomal acyl-CoA synthetase for the ABCD transporter import cycle, as demonstrated previously [7–9], but inconsistent with the ability of *faa2Δ* cells to oxidise myristate in intact cells (see Discussion).

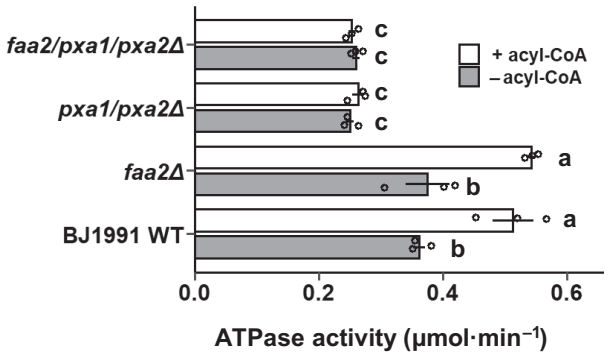


**Fig. 2.** Substrate-stimulated ATPase activity in isolated yeast peroxisomes. ATPase activity was measured in peroxisomes isolated from wild-type, *faa2Δ*,*pxa1/pxa2Δ*and*pxa1/pxa2/faa2Δ*cells in the presence or absence of acyl-CoA. Activity is normalised to 1 unit of peroxisomal marker enzyme 3-HAD. Values are the means ± SE ($n$ = 3). Letters indicate statistically significant differences (Tukey's HSD test; $P \leq 0.01$).

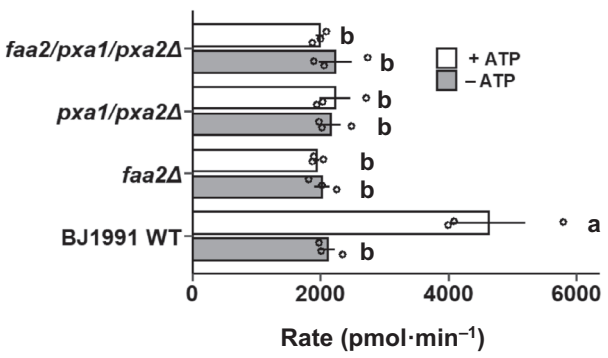


**Fig. 3.** [$^{14}$C]Myristoyl-CoA β-oxidation by isolated yeast peroxisomes. Isolated peroxisomes were incubated with [1-$^{14}$C]-myristoyl-CoA in the presence and absence of ATP, and β-oxidation rates were measured and normalised to 0.4 units of the peroxisomal marker enzyme 3-HAD. Values are the means ± SE ($n$ = 3). Letters indicate statistically significant differences (Tukey's HSD test; $P \leq 0.01$).

### Free CoA is released in peroxisomes of wild-type cells

To investigate the fate of the CoA moiety in peroxisomes and to distinguish this from metabolism of the fatty acyl moiety, we synthesised myristoyl-[$^3$H]-CoA for use in transport assays (Materials and methods). Following incubation of isolated peroxisomes with myristoyl-[$^3$H]-CoA and ATP, the assay mixture was applied to a G75 column to separate peroxisomes from the medium (Fig. S3). Figure 4A shows uptake of label derived from myristoyl-[$^3$H]-CoA by wild-type peroxisomes, which was markedly reduced in *pxa1/pxa2Δ* and *pxa1/pxa2/faa2Δ*. This suggests that in wild-type peroxisomes, the CoA moiety of myristoyl-CoA is imported into peroxisomes by Pxa1/Pxa2p and not released in the cytosol. The uptake of [$^3$H] was decreased in *faa2Δ* peroxisomes relative to wild-type. To investigate further the fate of imported CoA, free CoA was separated from acyl-CoA and acetyl-CoA using a C18 reverse phase column (Fig. S2). Figure 4B shows that in wild-type peroxisomes, 90% of imported acyl-CoA was hydrolysed to release free CoA, which is in agreement with reports that ABCD transporters have intrinsic ACOT activity [8–10]. The [$^3$H]acyl-CoA plus [$^3$H]acetyl-CoA contents of mutant peroxisomes were not statistically significantly different to that of wild-type, whereas [$^3$H]CoA was markedly reduced. Taken together, these measurements indicate that a [$^3$H]CoASH moiety from myristoyl-[$^3$H]CoA is transported *via* the Pxa1/Pxa2p heterodimer across the peroxisomal membrane and delivered to the peroxisomal lumen.

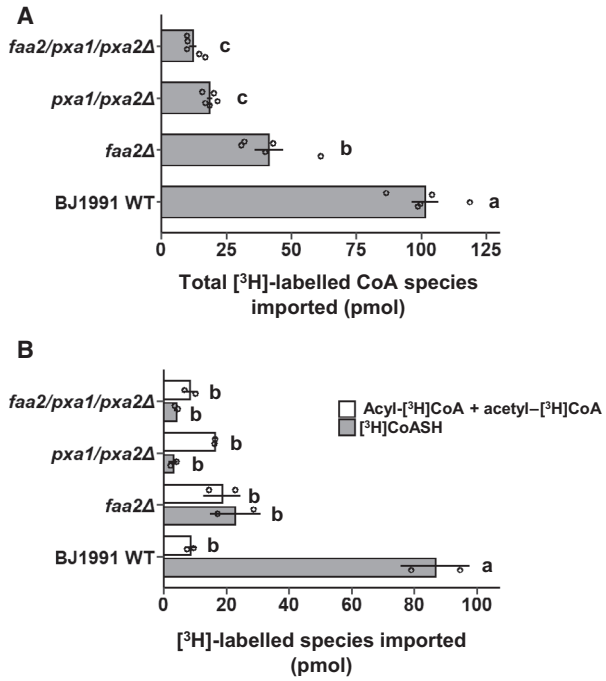


**Fig. 4.** The CoA moiety of fatty acyl-CoA is imported by yeast peroxisomes. Isolated peroxisomes from wild-type, *pxa1/pxa2Δ*, *faa2Δ*and*pxa1/pxa2/faa2Δ*cells were incubated with myristoyl-[$^3$H] CoA and ATP. After incubation, the medium was separated from the peroxisomes by size exclusion chromatography and$^3$H in different molecular species was quantified. Assays were normalised for peroxisome amount using 0.4 units of 3-HAD activity. (A) Total$^3$H (acyl-[$^3$H]CoA, acetyl-[$^3$H]CoA and [$^3$H]CoASH) imported into peroxisomes. Values are the means ± SE of five independent experiments; (B)$^3$H in acyl-[$^3$H]CoA and [$^3$H]CoASH fractions. Values are the means ± SD of two independent experiments. Letters indicate statistically significant differences (Tukey's HSD test; $P \leq 0.01$).

## Discussion

The transfer of CoA across the peroxisome membrane is essential to supply several CoA-dependent metabolic reactions involved in fatty acid β-oxidation and the glyoxylate cycle (Fig. 5; [2,3,36]). In this study, we employed a combination of genetics and biochemistry to investigate the role of ABCD in peroxisomal CoA homeostasis. ABCD proteins are well known to mediate peroxisomal import of β-oxidation substrates such as FA but potentially also contribute to the CoA pool [4]. β-oxidation measurements with intact yeast cells confirmed previous results [22] that the medium-chain fatty acid myristate is imported into yeast peroxisomes *via* two pathways: Pxa1/Pxa2p-independent uptake as free fatty acid requiring reactivation by the peroxisomal acyl-CoA synthetase, Faa2p, and Pxa1/Pxa2p-dependent uptake after formation of myristoyl-CoA in the cytosol (Figs 1A and 5). We found that isolated wild-type peroxisomes imported [1-$^{14}$C]-myristoyl-CoA (and myristoyl-[$^3$H]CoA) in an ATP- and Pxa1/Pxa2p-dependent manner (Figs 3 and 4), consistent with studies demonstrating ABCD-dependent import of long and very long-chain acyl-CoAs in trypanosomes or human peroxisomes [5,37]. The observation that there is a level of ATP- and genotype-independent β-oxidation in isolated peroxisomes (Fig. 3) is consistent with activity due to damaged peroxisomes and in line with the increase in β-oxidation of [1-$^{14}$C]-myristoyl-CoA seen in detergent-treated cell lysates compared to intact cells (Fig 1). In contrast, ATP-dependent [1-$^{14}$C]-myristoyl-CoA β-oxidation was absent in *faa2Δ* peroxisomes (Fig. 3), despite the finding that *faa2Δ* peroxisomes retained wild-type levels of acyl-CoA-stimulated ATPase activity (Fig. 2). This is in agreement with previous reports that reactivation inside the peroxisome is required [7,9]. However, our data from isolated peroxisomes differ from those from intact

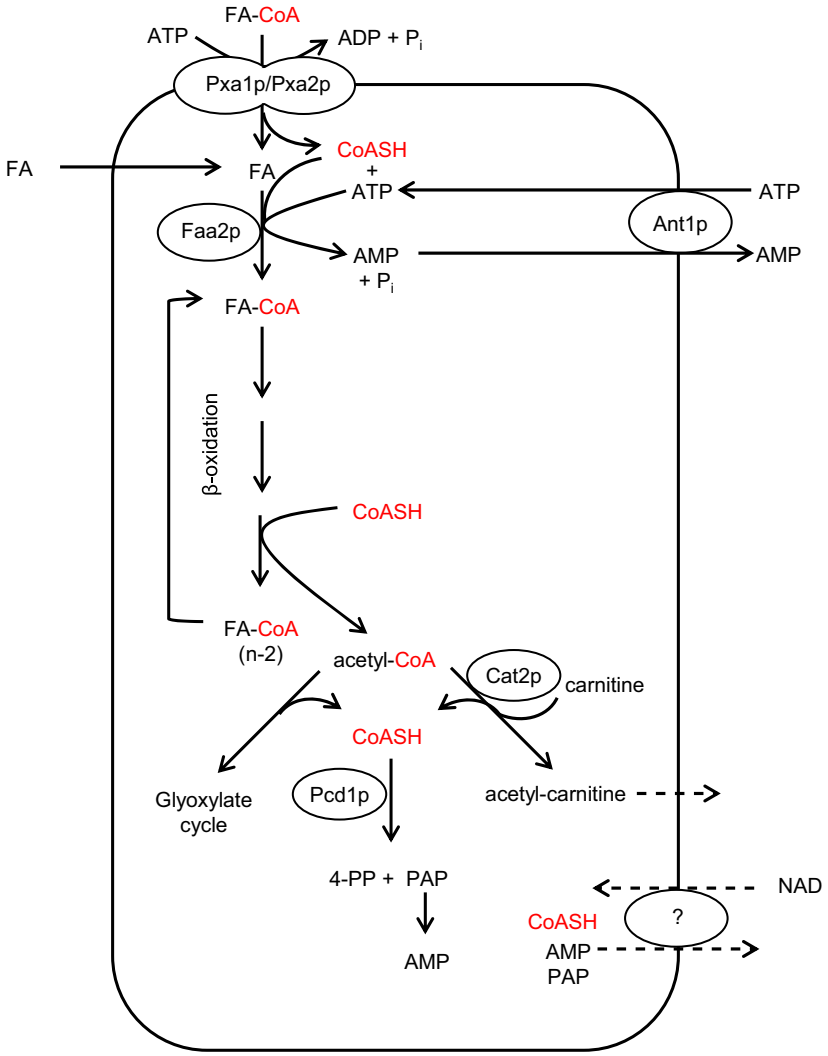


**Fig. 5.** The CoA budget in yeast peroxisomes. Revised model for import, consumption and regeneration of CoA during fatty acid β-oxidation, modified after [36,44]. FA are imported into the peroxisome*via* two routes: (a) Fatty acyl-CoAs (FA-CoA) are imported by the ABC transporter, Pxa1p/Pxa2p and the CoA moiety is cleaved off by intrinsic thioesterase activity of the transporter and released in the peroxisome lumen. (b) Free FA are imported independently of Pxa1/2p, and CoA esters are formed in the peroxisome*via*the action of the acyl-CoA synthetase, Faa2p (or by Fat1p for very long-chain FA; not shown). Thioester formation requires CoASH and ATP which is imported by the adenine nucleotide translocator, Ant1p. 'Core' β-oxidation involves the sequential actions of Fox1p (acyl-CoA oxidase), Fox2p (bifunctional 2-trans-enoyl CoA hydratase and hydroxyacyl-CoA dehydrogenase) and Fox3p (3-ketoacyl-CoA thiolase), to generate acetyl-CoA plus FA-CoA shortened by two carbons. Acetyl-CoA has two potential fates in yeast peroxisomes [24,45]: (a) It is stoichiometrically converted to CoASH in the glyoxylate cycle (but this is unlikely to operate in isolated peroxisomes since the cycle requires cytosolic aconitase and isocitrate lyase), and (b) it participates in the carnitine shuttle,*via*peroxisomal conversion of acetyl-CoA to acetyl carnitine, catalysed by carnitine acyltransferse, Cat2p and yielding CoASH [6]. Free CoA may be degraded by enzymes such as nudix hydrolases such as Pcd1p [39], to yield 3′-phosphoadenosine 5′-monophosphate (PAP) and 4′-phosphopantetheine (4-PP). A CoA carrier has not yet been identified for yeast, but may serve to exchange CoA, its metabolites and/or biosynthetic intermediates, by analogy with human SLC25A17. Dashed lines indicate transport steps which have not been confirmed experimentally. Figure reproduced from Refs. [6,24,36,39,44,45].

*faa2Δ* cells, in which myristate β-oxidation still occurs, albeit at a reduced level (Fig. 1A). The reason for this is unknown but the experimental systems are not directly comparable.

Transport assays with myristoyl-[$^3$H]CoA revealed that 90% of the label imported into wild-type peroxisomes was in the form of free CoA, which is consistent with thioesterase activity of the Pxa1/Pxa2p

transporter (Fig. 4B). Whilst it might be expected that CoASH and imported fatty acid would be rapidly re-esterified by Faa2p and then converted to shorter chain acyl-CoA esters and acetyl-CoA by β-oxidation, the tritiated CoASH will be diluted by the pre-existing pool of unlabelled CoASH. Therefore, labelled acyl-CoA species arising from downstream metabolism would be of low abundance immediately following release of [$^3$H]CoA in the peroxisome lumen. Interestingly, import and cleavage of myristoyl-[$^3$H]CoA were markedly reduced in *faa2Δ* peroxisomes, with only ~ 25 % free [$^3$H]CoA content compared to wild-type (Fig. 4B). This suggests that there is a small amount of import, but that transporter activity is impaired. Since free CoA and FA are the products of thioesterase activity, it is tempting to speculate that FA accumulate in the membrane of *faa2Δ* peroxisomes in the absence of a functional acyl-CoA synthetase and inhibit the thioesterase activity of Pxa1/Pxa2p, arresting the overall transport cycle. Product inhibition has been reported for other ACOT [38].

Although this study used yeast as a tractable experimental system, it is likely that the findings are applicable to plant and animal ABCD transporters. Cross-kingdom complementation experiments point to a shared role in import of substrates for β-oxidation and shared mechanism whilst indicating differences in substrate specificity [7–9,22,39–41]. In human fibroblasts, it has been asserted that acyl-CoA cleavage is not associated with import, since VLCFA-CoA β-oxidation by isolated peroxisomes is not dependent on addition of CoASH to the assay, whereas β-oxidation of free VLCFA requires exogenous CoASH [5]. However, the transport of CoA by the ABCD transporter, as proposed here for Pxa1p/Pxa2p, would explain the independence of VLCFA-CoA β-oxidation of exogenous CoASH in isolated peroxisomes. Importantly, human ABCD1 and ABCD2 only complement *pxa1/pxa2Δ* for β-oxidation of C22:0 and C24:0 in the presence of Faa2p, suggesting that cleavage does occur [7] and ACOT activity has in fact been demonstrated for recombinant human ABCD proteins [10].

The two import routes for free FA and fatty acyl-CoAs raise an important point about the peroxisomal CoA budget. β-oxidation and the glyoxylate cycle use CoA catalytically (Fig. 5); therefore, the import of a CoA moiety together with a fatty acid molecule does not require an additional source of CoASH and indeed may lead to CoA accumulation in the peroxisome. In contrast, free fatty acid import is dependent on the peroxisomal CoA pool which could be supplied by cleavage of imported acyl-CoAs or by a CoA carrier. The balance between free fatty acid and fatty acyl-CoA import will differ depending on the prevailing metabolic demands of the cell, and the CoA pool must be regulated accordingly. A CoA carrier has not yet been identified in yeast peroxisomes although the human MCF transporter PMP34/ANT1/SLC25A17 has been shown to exchange CoA, CoA biosynthetic intermediates and CoA salvage pathway metabolites *in vitro* [16]. Soluble peroxisomal thioesterases and nudix hydrolases also serve to regulate levels of acyl-CoA intermediates and free CoA levels to adapt to changes in pathway flux [42]. The yeast nudix hydrolase Pcd1p degrades CoASH to 3'-phosphoadenosine 5'-monophosphate (PAP) and 4′-phosphopantetheine [43]. It has been proposed that PAP could then be transported out of peroxisomes in exchange with $NAD^+$ (or CoA) or converted to AMP to function as exchange partner for $NAD^+$ (or CoA) import [3] (Fig. 5).

In conclusion, peroxisomal ABC transporters provide an import pathway for CoASH and can be viewed as part of regulatory unit also comprising coenzyme carriers and nudix hydrolases, which controls the peroxisomal CoA pool and tunes metabolism according to different metabolic demands. Future work will focus on identifying and characterising the remaining members of this regulatory unit and their integration.

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## Author contributions

CvR and LIJ conceived the study. CvR carried out the experimentation. CvR, AB, FLT and LIJ analysed data. CvR, LIJ, AB and FLT wrote the manuscript, which was critically read, revised and approved by all authors before submission.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Isolation of peroxisomes from wild type and mutant cells.

**Fig. S2.** Validation of C18 reverse phase column for the separation of free from esterified CoA.

**Fig. S3.** Myristoyl-[$^3$H]CoA uptake into isolated peroxisomes.