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# THE PEX5/PEX7- MEDIATED IMPORT OF PTS2-CONTAINING MATRIX PROTEINS: MECHANISTIC INSIGHTS ON PEX7 TRANSLOCATION AND RECYCLING STEPS

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**TABLE OF CONTENTS**

## PRECEITOS LEGAIS

O autor desta tese declara que interveio na concepção e execução do trabalho experimental, na interpretação e redacção dos resultados que, além de incluídos nesta tese, culminaram numa publicação internacional (artigo abaixo indicado), sob o nome de "**Rodrigues TA**". Além deste artigo, o autor declara ter também participado na elaboração de um artigo de revisão, no qual partilha a primeira autoria, e ainda de um capítulo de um livro do qual é primeiro autor.

The author of this thesis declares to have participated in the planning and execution of the experimental work, in the interpretation and preparation of the data which, besides being included in this thesis, were published in an international journal, under the name "**Rodrigues TA**". Moreover, the author declares to have also participated in the elaboration of a review paper, sharing the first authorship, and in a book chapter as first author.

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## SUMMARY/RESUMO

## SUMMARY

Peroxisomal matrix proteins are synthesized on cytosolic ribosomes and transported to the organelle by shuttling receptors. Matrix proteins containing a type 1 signal are carried to the peroxisome by PEX5, whereas those harboring a type 2 signal are recognized by PEX7 and then transported by a PEX5-PEX7 complex. The pathway followed by PEX5 during the protein transport cycle has been characterized in detail. After binding a cargo protein in the cytosol, PEX5 interacts with the peroxisomal docking/translocation machinery (DTM). Following this docking event, PEX5 gets inserted into the DTM acquiring a transmembrane topology, a step that results in the translocation of the cargo protein across the organelle membrane and its release into the peroxisomal lumen, all without ATP hydrolysis. PEX5 is then extracted from the DTM through a two-step mechanism. First, PEX5 is monoubiquitinated at a conserved cysteine (Cys 11 in human PEX5); this monoubiquitinated PEX5 species is subsequently dislocated from the DTM in an ATP-dependent manner by the two mechanoenzymes, PEX1 and PEX6, which compose the receptor export module (REM). Finally, ubiquitin is removed from PEX5 probably by a combination of enzymatic and non-enzymatic mechanisms. In contrast to the data available for PEX5, not much is known regarding mammalian PEX7. The little that is known regarding PEX7 derives mainly from studies in yeasts, organisms that possess a different architecture of the PEX7-mediated import pathway. In this work we have optimized a previously established peroxisomal in vitro import system to study the pathway followed by mammalian PEX7 during the PTS2 protein import cycle. We found that PEX7 reaches the peroxisome in a PEX5L- and PTS2-dependent manner where it acquires a protease-protected status. Acquisition of this status occurs upstream of the first cytosolic ATP-dependent step, *i.e.*, before ubiquitination of PEX5L. PEX7 passing through the peroxisome becomes partially exposed to the peroxisome matrix milieu suggesting that cargo release occurs at the trans side of the peroxisomal membrane. This *in vitro* system also allowed us to characterize the export step of PEX7. Our results show that whenever export of PEX5L is inhibited that of PEX7 is also blocked. This suggests that PEX7 exits the organelle through the DTM site occupied by PEX5L. Importantly, in vitro imported PEX5L and PEX7

display different export kinetics suggesting that their export is uncoupled. Additionally, exploring this PEX7 *in vitro* import/export system, we were able to obtain evidence suggesting that PEX7 travelling through the peroxisome is not completely released into the peroxisomal matrix. Finally, a putative link between cleavage of the PTS2 signal by Tysnd1, the signal processing peptidase, and PTS2 cargo-release into the peroxisomal lumen was discarded.

## RESUMO

As proteínas da matriz peroxissomal são sintetizadas em ribossomas livres no citosol e só depois transportadas para o organelo por receptores solúveis. As proteínas matriciais podem conter um sinal de endereçamento peroxissomal do tipo 1 (PTS1) sendo reconhecidas e transportadas pelo receptor PEX5, ou podem conter um sinal de endereçamento do tipo 2 (PTS2), sendo reconhecidas pelo receptor PEX7 e transportadas para o peroxissoma por um complexo PEX5-PEX7. O percurso seguido pelo receptor PEX5 durante o ciclo de importação de proteínas matriciais foi já amplamente descrito. Depois de ligar as proteínas-cargo no citosol, o receptor PEX5 interactua com a chamada maquinaria de “docking” e translocação (DTM). Após um primeiro passo de acoplagem, a PEX5 insere-se no DTM, adquirindo uma topologia transmembranar. Esta inserção resulta na translocação da proteína-cargo através da membrana e na sua libertação para o lúmen peroxissomal, tudo isto sem necessidade de hidrólise de ATP. A PEX5 é então extraída do DTM através de um processo que compreende dois passos. Primeiro, a PEX5 é monoubiquitinada numa cisteína conservada (Cys 11 na PEX5 humana); seguidamente, esta espécie monoubiquitinada é reconhecida e extraída do DTM pelas mecano-enzimas PEX1 e PEX6 num processo dependente de ATP. Finalmente, a molécula de ubiquitina é removida no citosol por desubiquitinases ou por um processo não enzimático. Tudo isto contrasta com o pouco que ainda se sabe sobre o receptor PEX7. Os dados disponíveis provêm de estudos em leveduras, organismos nos quais o sistema de importação mediado pela PEX7 apresenta uma arquitectura diferente da dos mamíferos. O trabalho apresentado nesta tese mostra que o receptor PEX7 só chega ao peroxissoma na presença de uma proteína-cargo e do receptor PEX5. Uma vez no peroxissoma, o receptor PEX7 adquire uma completa resistência ao tratamento com proteases exógenas. A entrada do receptor PEX7, bem como a libertação da proteína-cargo para a matriz, ocorre antes do primeiro passo dependente de ATP citosólico, ou seja, antes da monoubiquitinação do receptor PEX5. Mostramos também que o receptor PEX7, durante a sua passagem pelo peroxissoma, expõe pelo menos uma parte da sua cadeia polipeptídica à matriz peroxissomal o que sugere fortemente que a libertação da proteína-cargo se dá já

depois de atravessada a membrana do peroxissoma. Os dados descritos nesta tese sugerem também que a exportação do receptor PEX7 de volta para o citosol depende da exportação do receptor PEX5. No entanto, estes dois eventos apresentam cinéticas distintas sugerindo que os dois receptores deixam o organelo separadamente. Adicionalmente, são fornecidas evidências de que o receptor PEX7 nunca é libertado para a matriz do peroxissoma, ficando antes retida no DTM até a sua libertação para o citosol. Finalmente, este trabalho permitiu-nos também descartar uma possível conexão entre o processamento do sinal PTS2 na matriz peroxissomal e a libertação das proteínas PTS2 para a matriz peroxissomal.

## ABBREVIATIONS

| | |
|---|---|
| AAA | ATPases associated with diverse cellular activities |
| ABC | ATP-Binding Cassette |
| ACOX1 | Acyl-Coenzyme A oxidase 1 |
| ADHAPS | Alkyl-DHAP synthase |
| AGT | Alanine glyoxylate aminotransferase |
| ALDP | Adrenoleukodystrophy protein |
| AMACR | 2-methylacyl-CoA racemase |
| BSA | Bovine serum albumin |
| CHO | Chinese hamster ovary |
| DBP | D-bifunctional protein |
| DECR2 | 2,4-dienoyl-CoA reductase |
| DHAP | Dihydroxyacetone phosphate |
| DHAPAT | DHAP acyltransferase |
| DTM | Docking/translocation module |
| DTT | Dithiothreitol |
| DUB | Deubiquitinating enzyme |
| E1 | Ubiquitin-activating enzyme |
| E2 | Ubiquitin-conjugating enzyme |
| E3 | Ubiquitin ligase |
| E-64 | N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide |
| EDTA | Ethylenediamine tetraacetic acid |
| ER | Endoplasmic reticulum |
| FIS1 | Fission 1 |
| GFP | Green fluorescent protein |
| GSH | Glutathione |
| GST | Gluthathione-S-transferase |
| IgG | Immunoglobulin G |
| MFF | Mitochondrial fission factor |
| MOPS | 4-morpholinepropanesulfonic acid |
| mPTS | Membrane peroxisomal targeting signal |
| NALD | Neonatal Adrenoleukodystrophy |
| NTP | Nucleoside triphosphate |
| PAGE | Polyacrylamide gel electrophoresis |

| | |
|---|---|
| PBDs | Peroxisome biogenesis disorders |
| PEX | Peroxin |
| PHYH | Phytanoyl-CoA hydroxylase |
| PIM | Peroxisomal import machinery |
| PMP | Peroxisome membrane protein |
| PMSF | Phenylmethylsulfonyl fluoride |
| PNS | Postnuclear supernatant |
| PTS1 | Peroxisomal targeting signal 1 |
| PTS2 | Peroxisomal targeting signal 2 |
| REM | Receptor export module |
| RING | Really interesting new gene |
| SCP2 | Sterol carrier protein 2 |
| SCPx | Sterol carrier protein x |
| SDS | Sodium dodecylsulfate |
| SEM | Buffer containing sucrose, EDTA and MOPS |
| SH3 | Src homology 3 domain |
| TPR | Tetratricopeptide repeats |
| Tris | Tris(hydroxymethyl)aminomethane |
| Ub | Ubiquitin |
| Ub-PEX5 | Monoubiquitinated PEX5 |
| X-ALD | X-linked Adrenoleukodystrophy |
| WD | Tryptophan-aspartate repeat |
| ZS | Zellweger syndrome |
| ZSDs | Zellweger spectrum disorders |

# I- INTRODUCTION

## 1. Structure and function of peroxisomes

Peroxisomes are present in virtually all eukaryotic cells. They were first described in 1954 as microbodies by Rhodin (1), but their role remained elusive for well over a decade. The name “peroxisome” was proposed in 1966 by Christian de Duve, when he identified the first peroxisomal enzymes, namely hydrogen peroxide-originating oxidases and catalase, an enzyme that catalyses hydrogen peroxide disproportionation (2). These organelles are, unlike chloroplasts and mitochondria, surrounded by a single phospholipid bilayer membrane and are devoided of DNA (3–5). Their matrix is characterized by high protein concentrations. In some organisms and tissues (*i.e.* liver cells in rodents) some proteins are so abundant that they originate electron-dense crystalline structures (5–7) (see Figure 1). Peroxisomes are typically spherical organelles with a diameter ranging between 0.1 and 1 μm, though their morphology, abundance and even function can differ greatly between species, tissue and prevailing environmental conditions (reviewed in (8–11)).

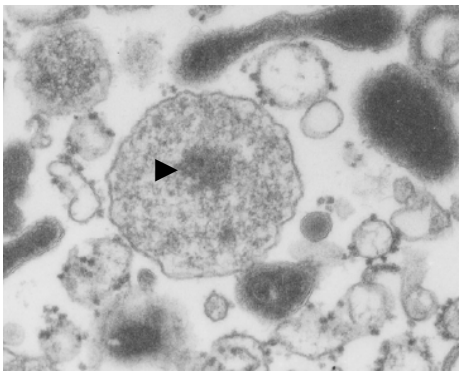

**Figure 1. Electron micrograph of peroxisomes from a rat liver postnuclear supernatant**. A crystalline inclusion of urate oxidase is marked (►). Micrograph kindly taken by Prof. Dr. Manuel Teixeira da Silva, IBMC, Porto, Portugal).

The biochemical plasticity of peroxisomes is so large that, in some organisms, specialized forms of this organelle received other designations. For instance, peroxisomes in some germinating seed cells are called glyoxysomes because they harbor enzymes of the glyoxylate cycle (12), Similarly, trypanosome peroxisomes are called glycosomes because they contain part of the glycolysis pathway (13). Finally, filamentous ascomycetes (*e.g. Neurospora crassa*) have Woronin bodies. These organelles are somewhat unusual as their only

established function is to plug septal pores in the event of hyphal damage. This prevents the loss of cytoplasm and, therefore, contributes to the maintenance of cellular integrity (14).

Despite the great functional variability of peroxisomes (15–17), two conserved metabolic pathways are found amongst evolutionary diverse organisms. These are the β-oxidation of fatty acids and hydrogen peroxide-degradation (18–20). In many yeasts/fungi and plants, the fatty acid β-oxidation pathway is exclusively localized in peroxisomes (21), whereas in mammalian cells this pathway occurs in both peroxisomes and mitochondria (21, 22). The enzymes of the peroxisomal β-oxidation pathway are also involved in the synthesis of chemical compounds which function as phytohormones in plants, such as jasmonates (23) or indol-3-acetic acid (16, 24, 25). Other peroxisome functions include the α-oxidation of branched-chain fatty acids in mammals and plants (26, 27), the main reactions of photorespiration in leaf peroxisomes (28), the final steps of penicillin biosynthesis in some filamentous fungi (29), or synthesis of bile acids (30) and ether lipids such as plasmalogens in mammals, which constitute more than 80% of the phospholipid content of the white matter in the brain and are thought to be involved in cell signalling and protection against reactive oxygen species (ROS) (31, 32). Peroxisomes are part of a coordinated multi-organelle cellular machinery, physically and functionally interacting with other subcellular compartments, namely mitochondria and the endoplasmic reticulum (ER) (33–38).

## 2. Peroxisomal Disorders

The importance of peroxisomes in human health and development is dramatically illustrated by a group of genetic diseases, the peroxisomal disorders, in which peroxisome functions are partially or even completely impaired. These diseases are either caused by defects in peroxisomal enzymes/transporters (single enzymes deficiencies) or by mutations in genes encoding peroxins, proteins that are specifically required for peroxisome maintenance and inheritance (the peroxisomal biogenesis disorders; PBDs, reviewed in (39–42))

## 2.1. Single enzyme deficiencies

The single peroxisomal enzyme deficiencies can be divided into distinct subgroups based on the affected peroxisomal metabolic pathway. They encompass single defects in peroxisomal ß-oxidation, plasmalogen biosynthesis and α-oxidation, among others (see Table 1). The most prevalent single enzyme deficiency is X-linked adrenoleukodystrophy (X-ALD), a progressive neurodegenerative disorder caused by defects in ALDP (a peroxisomal membrane protein of the ATP-Binding Cassette (ABC) family of transporter proteins) (43–45). This deficiency results in the accumulation of very long chain fatty acids in plasma and tissues and leads to the progressive demyelination of the central nervous system (reviewed in (46)).

**Table 1. Peroxisomal single enzyme deficiencies.**

| Peroxisomal pathway affected | Peroxisomal disease | Enzyme defect |
|---|---|---|
| **Ether phospholipid synthesis** | Rhizomelic chondrodysplasia punctata Type 2 (DHAPAT deficiency) | DHAP acyltransferase (DHAPAT) |
| | Rhizomelic chondrodysplasia punctata Type 3 (alkyl-DHAP synthase deficiency) | Alkyl-DHAP synthase (ADHAPS) |
| **Peroxisomal β-oxidation** | X-linked adrenoleukodystrophy (ALDP deficiency) | Adrenoleukodystrophy protein (ALDP) |
| | Acyl-CoA oxidase deficiency | Acyl-COA oxidase-1 (ACOX1) |
| | D-bifunctional protein deficiency | D-Bifunctional protein (DBP) |
| | 2-MethylacylCoA racemase deficiency | 2-MethylacylCoA racemase (AMACR) |
| | Sterol carrier protein X deficiency | Sterol carrier protein X (SCPx) |
| **Peroxisomal α-oxidation** | Refsum disease (phytanoyl-CoA hydroxylase deficiency) | Phytanoyl-CoA hydroxylase (PHYH) |
| **Glyoxylate detoxification** | Hyperoxaluria Type 1 (AGT deficiency) | Alanine:glyoxylate aminotransferase (AGT) |
| **$H_2O_2$-metabolism** | Acatalasaemia (CAT deficiency) | Catalase (CAT) |
| **Bile acid synthesis** | Bile acid-CoA:amino acid N-acyltransferase deficiency | Bile acid-CoA:amino acid N-acyltransferase (BAAT) |

Adapted from (40)

## 2.2. Peroxisomal biogenesis disorders

Peroxisomal biogenesis disorder (PBDs) are caused by mutations in *PEX* genes (47, 48). These genes encode many of the proteins required for peroxisome biogenesis, maintenance and inheritance, the so-called “Peroxins” (see below). Contrary to the single enzyme deficiencies, where only one metabolic pathway is affected, PBDs result in the loss of several, sometimes all, peroxisomal functions and, in some cases, in the complete absence of the organelle (reviewed in (47–49)).

The PBD group comprises the Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and rhizomelic chondrodysplasia punctata (RCDP) type 1. After the discovery that mutations in the same gene could lead to any of the first three conditions, they are presently collectively called the Zellweger spectrum disorders (ZSDs) that constitute a triad of overlapping disorders with ZS being the most severe, followed by NALD and IRD (48). Liver disease, variable neurodevelopment delay, retinopathy, and perceptive deafness with onset in the first months of life are clinical symptoms common to all three. In addition, patients with ZS are severely hypotonic at birth and have distinct facial features, peri-articular calcifications, severe brain dysfunction associated with neuronal migration defects and generally, the child does not survive past the first year (50). Patients with NALD have hypotonia, seizures, progressive white matter disease, and usually die in late infancy (42, 51). Patients with IRD may have external features reminiscent of ZS, but no neuronal migration disorder and no progressive white matter disease. The cognitive and motor development vary between a severe global handicap and moderate learning disabilities with deafness and visual impairment, due to retinopathy (26, 42, 52).

The Zellweger spectrum disorders have been associated with mutations in 13 different genes. Twelve of these genes encode peroxins involved in protein import to the peroxisomal matrix and membrane; severe mutations in these genes lead to the impairment of most, if not all, peroxisomal functions (39, 47, 53). Recently, a novel and milder form of the disease was identified, Interestingly, the only patient identified to date with this disease lacked the common biochemical indicators of a ZSD (54). Instead, immunofluorescence microscopy revealed abnormalities in peroxisome morphology: Peroxisomes were elongated or

enlarged and frequently arranged in rows which suggested a fission defect. Indeed, the genetic defect was found to be a homozygous point mutation in *PEX11β,* a gene involved in peroxisome division/proliferation (see section 3.1).

RCDP type 1 is clinically quite different from the Zellweger spectrum disorders although there are some shared features, including the cranial, facial (broad low nasal bridge, high arched palate, and dysplastic external ears), and ocular abnormalities. It is characterized by the shortening of the proximal bones, stippled epiphyses, and by growth and mental retardation (55–57). Besides the clinical differences, their genetics is also very distinct. While the ZSD can be caused by mutations in any of 13 genes referred to above, RCDP type 1 is attributed to mutations in a single gene, the *PEX7* gene (55, 57), disrupting only the function of a smaller subset of matrix proteins, those containing a peroxisomal targeting signal type 2 (see below).

**Table 2. PEX gene defects and clinical phenotypes in PBDs.**

| Gene | Phenotype | Pathway affected |
|---|---|---|
| **PEX1** | ZS, NALD, IRD | Matrix protein import (Receptor recycling) |
| **PEX2** | ZS, IRD | Matrix protein import (Receptor recycling) |
| **PEX3** | ZS | Membrane protein import |
| **PEX5** | ZS, NALD | Matrix protein import |
| **PEX6** | ZS, NALD | Matrix protein import (Receptor recycling) |
| **PEX7** | RCDP type1 | Matrix protein import |
| **PEX10** | ZS, NALD | Matrix protein import (Receptor recycling) |
| **PEX11β** | mild ZSD | Peroxisome proliferation/division |
| **PEX12** | ZS, NALD, IRD | Matrix protein import (Receptor recycling) |
| **PEX13** | ZS, NALD | Matrix protein import (Docking/Translocation) |
| **PEX14** | ZS | Matrix protein import (Docking/Translocation) |
| **PEX16** | ZS | Membrane protein import |
| **PEX19** | ZS | Membrane protein import |
| **PEX26** | ZS, NALD, IRD | Matrix protein import (Receptor recycling) |

Adapted from (47, 49, 58).

## 3. Peroxisome biogenesis

As previously mentioned, many of the proteins required for peroxisome biogenesis are encoded by the so-called *PEX* genes and are commonly designated by peroxins. They are referred to as PEX*n*, where *n* is a number that reflects the chronological order of their discovery. Presently, more than 30 peroxins have been identified in the several organisms used to study peroxisome biology (Table 3). However, the number of PEX proteins phylogenetically conserved is much lower. In mammals, for instance, only 16 peroxins are presently known. Despite this phylogenetic variability, it is now evident that the basic aspects of peroxisome biogenesis have been well conserved throughout evolution (59–61). Peroxins are involved in three key stages of peroxisome biogenesis, namely, import of peroxisomal membrane proteins, import of peroxisomal matrix proteins and peroxisomal proliferation, as detailed below.

### 3.1. Origin and maintenance of the peroxisomal membrane

The origin of the peroxisomal membrane has always been a matter of much debate in the field (62, 63). The initial structural studies, based on electron microscopy, suggested that peroxisomes were generated by budding from the ER (63, 64). However, subsequent biochemical data have shown that several peroxisomal membrane proteins (PMPs) are synthesized on free ribosomes in the cytosol and posttranslationally imported into pre-existing peroxisomes (8, 65, 66), an observation behind the classic “growth and division model” according to which peroxisomes arise solely by fission and subsequent growth of the pre-existent organelle. Nevertheless, considering that most membrane lipids are synthesized in the ER, there is little doubt that this organelle participates in the biogenesis of the peroxisomal membrane. In fact, the lipid composition of the peroxisomal membrane resembles that of the ER, being mostly comprised of phosphatidylcholine and phosphatidylethanolamine (67). Although several researchers in the field continue to propose that pre-peroxisomal vesicles are generated by a budding mechanism out of the ER (68–71), much of the data supporting this perspective has been recently questioned ((72)**;** see also below).

Furthermore, there are data suggesting that the “ER-to-peroxisome” lipid transport occurs not through vesicular trafficking, but rather through close physical contact between both organelles, a finding again supporting the growth and division model (73–77).

**Table 3. Proteins implicated in peroxisomal biogenesis (peroxins).**

| | PEX protein | Localization | Organism | Properties / Functions |
|---|---|---|---|---|
| **Matrix protein import** | **PEX1** | Cyt / Memb | M, P, F, Y | Receptor export; AAA ATPase |
| | **PEX2** | Memb | M, P, F, Y | E3; RING zing-binding domain |
| | PEX4 | Memb/Cyt | P, F, Y | E2 |
| | **PEX5** | Cyt / Memb | M, P, F, Y | PTS1 and PTS2 targeting; IDD, TPRs |
| | **PEX6** | Cyt / Memb | M, P, F, Y | Receptor export; AAA ATPase |
| | **PEX7** | Cyt / Memb | M, P, F, Y | PTS2 targeting; adaptor protein; WD repeats |
| | PEX8 | Matrix/ Memb | F, Y | |
| | **PEX10** | Memb | M, P, F, Y | E3; RING zing-binding domain |
| | **PEX12** | Memb | M, P, F, Y | E3; zing-binding domain |
| | **PEX13** | Memb | M, P, F, Y | SH3 |
| | **PEX14** | Memb | M, P, F, Y | Coiled-coil |
| | PEX15 | Memb | F, Y | PEX1/PEX6 membrane anchor |
| | PEX17 | Memb | Y | Coiled-coil |
| | PEX18 | Cyt / Memb | Y | PTS2 targeting |
| | PEX20 | Cyt / Memb | F, Y | PTS2 targeting |
| | PEX21 | Cyt / Memb | Y | PTS2 targeting |
| | PEX22 | Memb | P, F, Y | PEX4 membrane anchor |
| | **PEX26** | Memb | M, F, Y | PEX1/PEX6 membrane anchor |
| | PEX33 | Memb | F | Coiled-coil |
| **Membrane protein import** | **PEX3** | Memb | M, P, F, Y | |
| | **PEX16** | Memb | M, P, F, Y | |
| | **PEX19** | Cyt / Memb | M, P, F, Y | PMP targeting; Farnesylation motif |
| **Proliferation and inheritance** | **PEX11** | Memb | M, P, F, Y | |
| | PEX23 | Memb | F, Y | Dysferlin |
| | PEX24 | Memb | F, Y | |
| | PEX25 | Memb | Y | |
| | PEX27 | Memb | Sc | |
| | PEX28 | Memb | Sc | |
| | PEX29 | Memb | Y | |
| | PEX30 | Memb | Sc | Dysferlin |
| | PEX31 | Memb | Sc | Dysferlin |
| | PEX32 | Memb | Y | Dysferlin |
| | PEX34 | Memb | Sc | |

Adapted from (78). Mammalian peroxins are highlighted in blue and bold. Abbreviations: Cyt, cytosol; Memb, membrane; M, mammals; P, plants; F, fungi; Y, yeast; Sc, *S. cerevisiae*; PTS1, peroxisomal targeting signal 1; PTS2, peroxisomal targeting signal 2; IDD, intrinsic disordered domain; TPRs, tetratricopeptide repeats; WD, Tryptophan-aspartate repeat; SH3, Src homology 3; RING, really interesting new gene; E3, ubiquitin ligase; Ub, ubiquitin; AAA, ATPases associated with diverse cellular activities; E2, ubiquitin-conjugation enzyme; PMP, peroxisomal membrane protein.

Many of the components that constitute the peroxisome proliferation, elongation and fission machinery have already been identified in mammals, yeast and plants (79–85). Chief among these is PEX11, one of the first elements of this machinery to be described, and one that is present in all eukaryotic organisms (86, 87). In mammals there are three isoforms of this peroxin, each encoded by a different gene (83, 88, 89): PEX11α whose expression is induced by peroxisome proliferating agents (response to external stimuli) (61, 90), PEX11β and PEX11γ which are constitutively expressed and thought to be responsible for the constitutive peroxisomal division (91, 92). PEX11 was shown to have the ability to reshape and elongate the peroxisomal membrane (93–95). Furthermore, PEX11 also seems to take part in the recruitment of other components of the peroxisomal fission machinery (96). In yeast, where there is only one *PEX11* gene, other structurally related peroxins, such as PEX25 and PEX27, come into play (97–99).

Interestingly, peroxisome share part of the fission machinery with mitochondria (90, 100–103). The division of peroxisomal membranes requires the dynamin-related protein DLP1, a self-assembling GTPase with mechano-chemical properties known to tubulate and constrict membranes (92, 104, 105). Additionally, the membrane adaptors, Fission1 (FIS1) and the mitochondrial fission factor (MFF) were also found in peroxisomes. These proteins are believed to act as recruiting factors for DLP1 (100, 106, 107).

### 3.1.1. Import of peroxisomal membrane proteins

The machinery that targets newly synthesized peroxisomal membrane proteins (PMPs) to the organelle is completely different from the one sorting matrix proteins (see section 3.2). The first observation leading to this conclusion was the fact that in cells where the import of matrix proteins is defective, one could still find peroxisomal ghosts, *i.e.* membrane vesicles that although devoided of their matrix content, still possessed all peroxisomal membrane proteins (41, 108, 109). The import machinery of PMPs appears to comprise only 2, perhaps 3, peroxins. These are PEX19, PEX3 and, in some organisms, also PEX16 (110–114). PEX19 is a soluble protein capable of interacting with a broad variety of newly synthesized PMPs via their membrane peroxisomal targeting signal (mPTS) (115–120). It is currently believed that PEX19 acts as a chaperone for newly synthesized PMPs,

shielding their hydrophobic surfaces in the cytosol thus preventing aggregation (121). PEX19 also acts as the PMP import receptor, directing them to the peroxisomal membrane (115, 116, 122–125). PEX3 is thought to work as a membrane recruitment factor for cargo-loaded PEX19 (126–128). In fact, PEX3 has a higher affinity for cargo-loaded PEX19 than for PEX19 alone (128). It is possible that insertion of the PMP into the peroxisomal membrane is promoted by PEX3. Indeed, there are some data suggesting that the PEX19-interacting domain of PEX3 which is exposed to the cytosol, also interacts with membrane lipids, an interaction that is modulated by PEX19 itself (129). Interestingly, the peroxisomal targeting of PMPs into the organelle membrane does not require ATP, suggesting that the thermodynamic driving force for the whole process may derive from the insertion of the PMP into the peroxisomal lipid bilayer (65, 128).

The role of PEX16 remains vastly unknown. Whereas mutation of the corresponding gene seems to lead to the absence of peroxisomal vesicles in some organisms (i.e. mammals (110)), in others, no marked effect could be observed (130, 131). In yeasts, with the exception of *Yarrowia lipolytica*, PEX16 orthologs have yet to be found (132).

Interestingly, cell lines/yeast strains lacking PEX3 or PEX19, in which peroxisomal membrane vesicles are seemingly absent (111, 112, 114, 133), are capable of generating peroxisomes after a functional copy of the corresponding gene is reintroduced (112, 113). These findings led to the belief that in addition to the fission of mature organelles there is also *de novo* synthesis of peroxisomes. Furthermore, there is some evidence suggesting that the integral peroxisomal membrane protein PEX3 is sorted to the peroxisomal membrane through the ER (134–136), an observation that has also been interpreted as evidence supporting the ER as the origin of the peroxisomal membrane and not just as a supplier of components to pre-existing peroxisomes. Recently, however, new data was found contradicting some of these earlier findings. It was shown that yeast cells lacking PEX3, already contain vesicular structures that harbour components of the docking/translocation machinery, PEX13 and PEX14, among a few other peroxisomal proteins. Additionally, when PEX3 was re-introduced, this peroxin was sorted, not through the ER, but directly to these vesicular structures, which subsequently mature into normal peroxisomes (72). Clearly, the data regarding the *de novo* formation of peroxisomes are to be interpreted with great caution and the

physiological relevance of this process and its overall contribution to peroxisomal dynamics in normal cells remain to be determined (71, 137–139).

### 3.2. Matrix protein import

Peroxisomes do not contain DNA and, as such, all peroxisomal matrix proteins are encoded in the nucleus, synthesized on cytosolic ribosomes and posttranslationally imported peroxisomes. The majority of peroxins (10 out of 16 peroxins present in mammals) are components of the peroxisomal matrix protein import machinery (PIM). Collectively these proteins ensure the correct delivery of newly synthesized proteins to the organelle lumen. Most mammalian PIM peroxins are part of one of two functional/structural units: PEX13, PEX14, and the “Really Interesting New Gene” (RING) peroxins PEX2, PEX10, and PEX12 compose the membrane-embedded docking/translocation module (DTM; (140–142)); the two peroxisomal “ATPases associated with diverse cellular activities” (AAA ATPases), PEX1 and PEX6, together with their peroxisomal membrane anchor, PEX26, comprise the receptor export module (REM; (143, 144)). The peroxisomal protein shuttling receptors PEX5 and PEX7 complete the list of mammalian peroxins that integrate the PIM (55, 145, 146). In addition to these peroxins, the mammalian PIM also comprises other proteins, which are mostly involved in ubiquitination/deubiquitination events (see below). Due to the fact that their function is not restricted to the PIM they are not classified as peroxins.

The mammalian peroxins referred to above have orthologs in all peroxisome-containing organisms characterized so far, from yeasts and fungi to plants (60, 61). Strikingly, however, the reverse is not true. Indeed, several peroxins found in plants and lower eukaryotes do not exist in mammals. Apparently, evolution led to simpler PIMs. At least two different mechanisms seem to be behind this simplification. In one case, the function of two yeast/fungi/plant peroxins, PEX4 and PEX22, ended up being carried out by a family of mammalian ubiquitin-conjugating enzymes (E2D1/2/3) involved in many other pathways (147). In another case, a peroxin (PEX5) acquired the capacity to perform two different tasks (import of both PTS1- and PTS2-containing proteins; see below) (148–151), each of which is performed by a different peroxin in yeasts/fungi (see (152), and references cited therein). Despite these differences, the basics of the mechanism

of protein import into the peroxisomal matrix remained relatively well conserved during evolution (16, 153–156).

### 3.2.1. The PEX5-mediated peroxisomal matrix protein import pathway

The import pathway of newly synthesized proteins into the matrix of the organelle is generally described using a PEX5-centered perspective comprising 8 different stages/sub-stages (see Figure 2): First, free PEX5 (stage 0 PEX5) binds newly synthesized peroxisomal matrix proteins in the cytosol originating “stage 1a PEX5”; the receptor-cargo complex then docks at DTM (stage 1b). This interaction ultimately results in the insertion of PEX5 into the DTM (stage 2 PEX5), with the concomitant translocation of the cargo into the peroxisomal lumen; PEX5 is then monoubiquitinated (stage 3a PEX5) and consequently recognized by the receptor export module (stage 3b PEX5). Monoubiquitinated PEX5 is then extracted from the DTM (stage 4a PEX5). Finally, after release into the cytosol, monoubiquitinated PEX5 (stage 4b PEX5) is deubiquitinated, thus freeing PEX5 for another round of import (stage 0 PEX5). Interestingly, the steps that culminate in the translocation of the cargo protein across the peroxisomal membrane occur all in an ATP-independent manner. In contrast, the steps necessary to reset this protein transportation system require ATP consumption. A detailed description of each one of these steps is presented below.

#### *Cargo recognition by the shuttling receptors PEX5 and PEX7*

Peroxisomal matrix proteins are targeted to the organelle because they possess one of two types of peroxisomal targeting signals (PTS). The vast majority of them harbor a PTS type 1 (PTS1), a tripeptide with the sequence S-K-L, or a conserved variant, present at their extreme C termini (157, 158). A few peroxisomal matrix proteins possess instead a PTS2. This is an N-terminal degenerated nonapeptide with the consensus sequence (R/K)-(L/V/I)-$X_5$-(H/Q)-(L/A) (159–161). In mammals, only four proteins harboring a PTS2 have been identified, while in plants, PTS2-containing proteins may comprise up to one third of all peroxisomal matrix proteins (162). For some organisms (*e.g. Caenorhabditis elegans,* (163, 164)), this PTS2-mediated import is even completely absent. In contrast to the PTS1, which remains intact upon import, the PTS2 is generally

cleaved in the peroxisomal matrix of higher eukaryotes by a serine protease (Tysnd1 in mammals and DEG15 in plants) (165–167).

In mammals, plants and many other organisms, all peroxisomal matrix proteins are transported to the peroxisome by PEX5 (148–151). PEX5 is a 70-kDa monomeric protein which *in vivo* displays a dual subcellular localization, peroxisomal and cytosolic, reflecting its role as a shuttling receptor (168–170). Structurally, PEX5 comprises two domains (see Figure 3): 1) a natively unfolded N-terminal half that contains the binding site for PEX7, a highly conserved cysteine residue, and several pentapeptide diaromatic repeats (WXXXF/Y) necessary for a productive interaction with the DTM (148, 171–173); and 2) a structured C-terminal half possessing seven tetratricopeptide repeats (TPRs) domains that constitute the PTS1-binding site (174).

Interestingly, binding of PEX5 to newly synthesized matrix proteins that are oligomeric in their native state strongly inhibits their oligomerization, suggesting that PEX5 is also a chaperone/holdase (175). This property is probably crucial to avoid premature oligomerization in the cytosol of proteins that no longer expose their PTS1 upon oligomerization (176, 177). The interaction between PEX5 and PTS1 proteins is direct and sufficient to ensure that these proteins are efficiently targeted to the organelle. The interaction involves the PTS1 peptide on one side, and the TPR domains of PEX5 on the other, but the N-terminal half of PEX5 also contributes for the interaction (148, 175, 178–180). The interaction between PEX5 and PTS2-containing proteins requires PEX7, the cytosolic receptor that recognizes the PTS2 and serves as an adaptor for the PEX5-mediated import of PTS2 proteins (148–151). It is important to note that not all PEX5 molecules can drive import of PTS2 proteins. In mammals, the *PEX5* transcript undergoes alternative spicing to originate two major isoforms (148): the larger isoform of PEX5 (PEX5L), which seems to be predominantly expressed (172), and a smaller isoform (PEX5S), which lacks a 37-amino acid long insert that contributes for PEX7 binding. PEX5S is therefore unable to interact with this receptor and consequently unable to import PTS2 proteins.

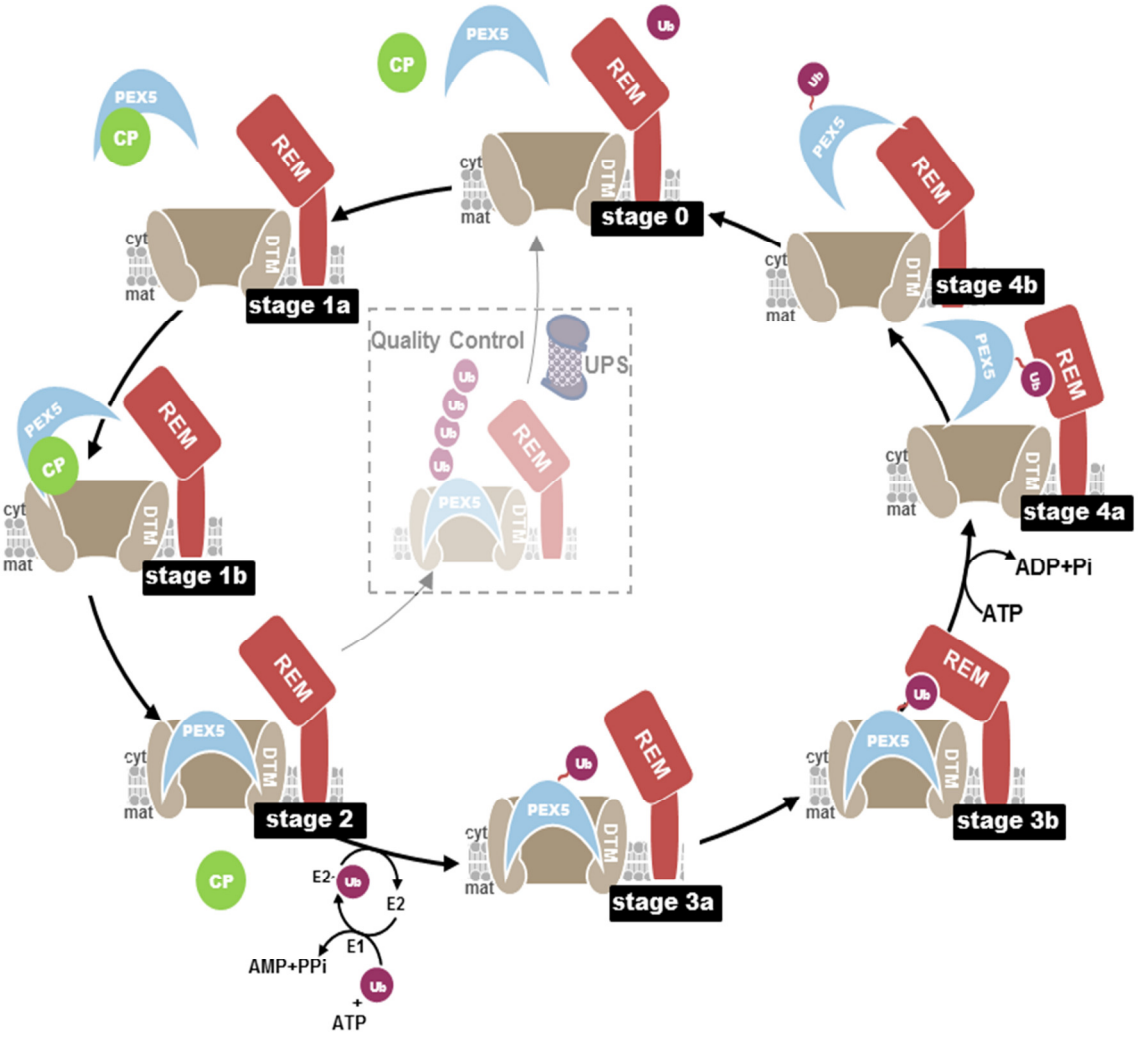


**Figure 2. The PEX5-mediated import pathway.**

Using a PEX5-centered *in vitro* import system, five different populations of PEX5 could be detected (stages 0 to 4, substages "a" and "b" are mostly of conceptual nature). Peroxisomal matrix cargo proteins (CP) are recognized by free cytosolic PEX5 [*stage 0 PEX5*]. The receptor-cargo protein complex [*stage 1a PEX5*] docks at the peroxisomal membrane docking/translocation machinery yielding *stage 1b PEX5*. The strong protein-protein interactions established between the receptor and DTM components result in the insertion of the receptor into the DTM [*stage 2 PEX5*] and the concomitant translocation and release of the cargo protein into the organelle matrix. The receptor is then monoubiquitinated at a conserved cysteine residue [*stage 3 PEX5*], and extracted back to the cytosol by the ATP-dependent receptor export module (REM) originating monoubiquitinated PEX5 in the cytosol [*stage 4 PEX5*]. Finally, the ubiquitin moiety is rapidly removed by a combination of enzymatic (DUBs) and non-enzymatic steps (*e.g.*, by nucleophiles such as glutathione, GSH) yielding once again free PEX5 [*stage 0 PEX5*] that can now undergo another import cycle. A control mechanism may act on receptors that become jammed at the DTM. These receptors are polyubiquitinated, extracted and degraded via the ubiquitin-proteasome pathway (UPS).

PEX7 is a 35-kDa soluble protein of the WD40 repeat family (160, 181, 182). Its structure comprises six WD repeats (~40 amino acids motifs, containing a Trp(W)-Asp(D) dipeptide) that fold into a four-stranded antiparallel β-sheet domain (see figure 3). Although the N-terminal region of PEX7 lacks sequence similarity with other WD40 domains, it assumes a similar structure. PEX7 thus folds into a seven-bladed beta-propeller in a circularised structure, typical of many WD-repeat proteins (183). Early experiments aiming at defining the subcellular localization of PEX7 gave contradicting results, apparently depending on how the protein was epitope-tagged. In general, PEX7 with a tag at the N terminus was predominantly cytosolic (55, 181), while PEX7 with a COOH-terminal tag was localized to the peroxisome (182). Subsequent success in raising antibodies against PEX7 allowed an analysis of the endogenous protein. It was found that PEX7 is mainly cytosolic but a small fraction of the protein is also present in the peroxisome (184–186). This dual subcellular localization *in vivo* suggested that PEX7, like PEX5, functions as a shuttling receptor for newly synthesized PTS2 containing proteins.

In lower eukaryotes, PEX5 does not interact with PEX7, and therefore the receptor function of PEX5 is restricted to PTS1 proteins. In these organisms, targeting of PTS2 proteins is ensured by a species-specific co-receptor (PEX20, PEX18 or PEX21) which displays structural/functional similarities with the N-terminal half of mammalian PEX5, including the conserved cysteine residue and the capacity to interact with PEX7 and PEX14 (152, 160, 187–189).

Strikingly, a few peroxisomal matrix proteins lack either one of these PTSs but are nonetheless targeted to the organelle by PEX5. In some cases, these proteins seem to rely on some ill-defined internal PTSs that are recognized by the N-terminal half of PEX5 (179). It has also been suggested that these proteins may be imported “piggybacking” with other proteins that do harbor a peroxisomal targeting signal (190–193).

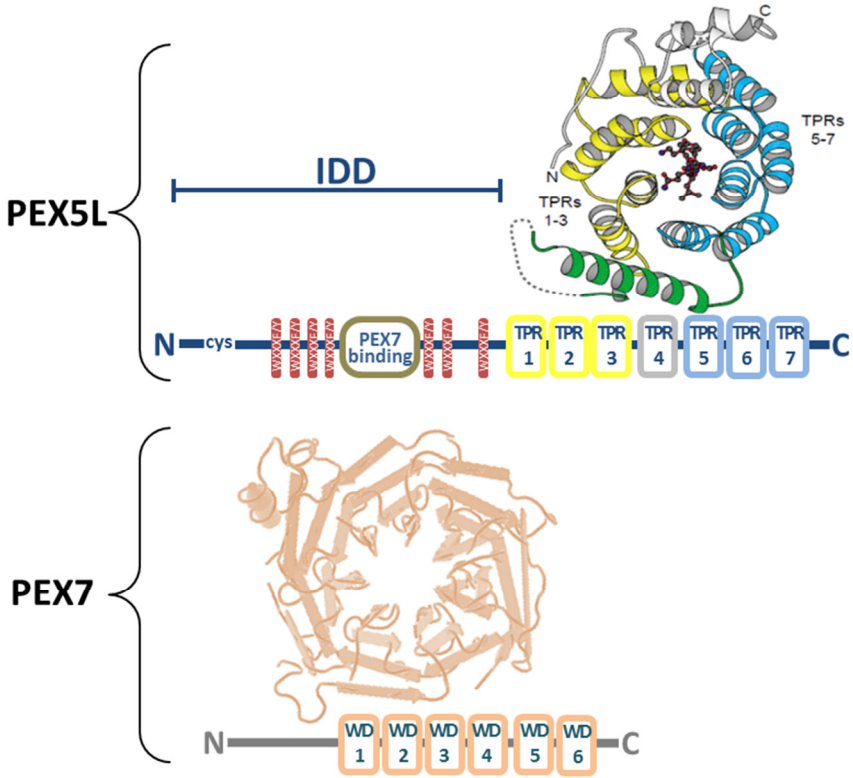


**Figure 3. Structure of mammalian PEX5L and PEX7.**
*Schematic representation of PEX5L.* IDD, intrinsically disordered domain. The N-terminally conserved cysteine is shown (cys); red bars indicate the diaromatic motifs (WXXXF/Y); the yellow, grey and blue boxes represent the seven TPR domains. The brown box indicates the PEX7 binding site that is present only in the large isoform of PEX5. Protein structure model of PEX5 taken from (174) (PDB: 1FCH). *Schematic representation of PEX7*. Orange boxes indicate the six WD repeats; Crystal structure of yeast PEX7 (PDB: 3W15, (183)).

### *Docking and translocation of the receptor-cargo complex into the DTM and cargo release*

Following cargo recognition, PEX5 interacts with the DTM. Of the DTM constituents, PEX13 and PEX14 are the most likely candidates to mediate this step (see ref. (194, 195)). Further characterization of the docking step showed that it could occur even at low temperatures (0 ºC) and that at this stage, the interaction between PEX5 and the DTM was still reversible (196). After docking, PEX5 becomes inserted into the DTM and the cargo protein is released into the peroxisomal matrix (196–198). Contrary to the docking step, translocation of PEX5 (and cargo protein) only occurs at higher temperatures (>16 ºC) and the interaction between PEX5 and the DTM becomes essentially irreversible ((196, 199), see below). PEX5 at this stage displays a transmembrane topology having

most of its polypeptide chain facing the peroxisomal matrix, whereas a 2-kDa N-terminal portion remains exposed to the cytosol (200, 201). Importantly, this insertion of PEX5 into the DTM is a cargo protein-dependent process (202). These observations are at the basis of the current model proposing that PEX5 pushes cargo proteins across the peroxisomal membrane as it gets inserted into the DTM (156, 197, 203). Remarkably, *in vitro* import experiments have shown that neither insertion of PEX5 into the DTM nor translocation of cargo proteins across the peroxisomal membrane are affected by non-hydrolyzable ATP analogs or by ATP depletion of the import assays (196, 198, 203). Likewise, ionophores have no effect on any of these events (198), in agreement with the fact that the peroxisomal membrane is readily permeable to small ions/molecules (204, 205). Apparently, the PIM uses neither the energy of ATP hydrolysis nor a membrane potential to transport proteins from the cytosol into the organelle matrix. Altogether, these findings led to the proposal that the driving force for the cargo protein translocation step resides in the strong protein-protein interactions that PEX5 establishes with components of the DTM (203).

***PEX5 monoubiquitination***

The first clue that ubiquitin should play some role in the peroxisomal protein import pathway dates back to 1992 when one of the yeast genes involved in this pathway was found to encode the ubiquitin-conjugating enzyme, PEX4 (206). The awareness, a few years later, that the three RING peroxins present in all peroxisome-containing organisms might well be members of a vast family of ubiquitin ligases E3s (207), fed this suspicion. However, the main mechanistic connection between ubiquitin and the PIM remained elusive for many years. As stated above, DTM-embedded PEX5 exposes approximately 2 kDa of its N terminus to the cytosol. This small N-terminal domain includes a cysteine-containing motif that is conserved not only in PEX5 proteins from all organisms, but also in the PTS2 co-receptors PEX20, PEX18 and PEX21. Interestingly, deletion of this domain or the substitution of the cysteine residue on these peroxins did not affect their capacity to get inserted into the DTM, but rendered them completely incompetent in the export step (199, 208–210). The significance of these observations finally became clear when PEX5 was found to be monoubiquitinated at this cysteine residue (211, 212). More recent data also

confirmed that the PTS2 co-receptors, PEX20 and PEX18, are also ubiquitinated at the conserved cysteine residue (213, 214).

Monoubiquitination of PEX5 is absolutely mandatory for the next step of this import pathway, the extraction of monoubiquitinated PEX5 back into the cytosol (211, 215). In agreement, yeast strains lacking PEX4 do not monoubiquitinate PEX5 (212), and are unable to recycle peroxisomal PEX5 back into the cytosol (215). Orthologs of both PEX4 (and PEX22, its membrane anchor) have been found using bioinformatic analyses in several yeasts/fungi and plants, suggesting that all these organisms have a peroxisomal E2 dedicated to the peroxisomal protein import pathway (60, 61, 216). Strikingly, however, no orthologs could be found in mammals and many other organisms. Proteomic studies aiming at identifying new mammalian peroxisomal proteins also failed to reveal the existence of a peroxisome-bound E2 (217–219). Remarkably, the E2 activity acting on mammalian PEX5 was found to be confined to the cytosol, and purification of this activity led to the identification of three almost identical cytosolic E2s, E2D1/2/3 (UbcH5a/b/c in humans) (147), a group of multipurpose E2s involved in many other biological pathways (220, 221).

As mentioned earlier, three of the five core components of the DTM are the RING peroxins, PEX2, PEX10 and PEX12 which strongly suggested that the DTM itself is the E3 ligase catalyzing PEX5 monoubiquitination. Interestingly, several studies suggest that PEX5 can still enter the DTM in cells lacking RING peroxins (140, 222, 223). Apparently, and similarly to multi-subunit E3s (224), the substrate-recruiting function of the DTM/E3 resides not in the RING peroxins but rather in other subunits of the complex (*e.g.* PEX14 and PEX13.) *In vitro* ubiquitination assays using recombinant RING $Zn^{2+}$-binding domains from yeast and plant RING peroxins have shown that they all display E3 activity when assayed with human UbcH5 (225, 226) or with yeast PEX4 (227). More recently, *in vitro* ubiquitination assays with the mammalians counterparts showed similar results for PEX10 and PEX2 (228). Interestingly, while PEX12 has no E3 activity *per se*, it stimulated the E3 activity of PEX10 (228). In principle, one RING peroxin alone could promote monoubiquitination of PEX5 at the conserved cysteine. However, recent *in vivo* data seem to suggest that the RING peroxins are not redundant and that all three together are required for receptor ubiquitination (214).

It is unclear why PEX5 is ubiquitinated at a cysteine residue instead of the more classical lysine residue (229). Substitution of the conserved cysteine residue in PEX5 by a lysine results in a seemingly normal protein that enters the DTM, receives a single ubiquitin molecule and is exported back into the cytosol as efficiently as the wild-type protein in *in vitro* assays (230). Furthermore, when expressed in embryonic fibroblasts from a *PEX5* knockout mouse, this PEX5 mutant protein is capable of reinstating peroxisomal protein import, as efficiently as the wild-type PEX5 protein (230). Nevertheless, some hypotheses regarding the conserved cysteine have been formulated. These include the possibility to deubiquitinate cytosolic Ub-PEX5 using a non-enzymatic mechanism (see below section “PEX5 deubiquitination”) or the potential to block the DTM under some conditions (*e.g.*, oxidative stress) through chemical modification of the conserved cysteine residue (*e.g.*, oxidation, glutathiolation, nitrosylation). In fact, a recent study demonstrated that protein import to the peroxisomal matrix is a redox-sensitive process, a property that was Cys 11-dependent. Additionally, this study also showed that PEX5, when exposed to oxidized glutathione, becomes unable to undergo monoubiquitination. Therefore, it was proposed that the conserved cysteine residue may function as a redox switch that regulates PEX5 activity in response to oxidative stress (231). Theoretically, obstruction of the DTM by export-incompetent PEX5 molecules would result in a cytosolic localization for newly synthesized peroxisomal enzymes (*e.g.*, catalase, epoxide hydrolase and glutathione S-transferase κ), a situation that might be advantageous under some stress conditions (see also (11, 230)).

***PEX5 dislocation to the cytosol***

As stated above, the interaction of DTM-embedded PEX5 (*i.e.* stage 2 PEX5) with components of this membrane module is essentially irreversible (199). Therefore, it is not surprising that extraction of the receptor back into the cytosol requires energy input. Indeed, it has been shown that extraction of Ub-PEX5 from the DTM requires ATP hydrolysis. It is presently believed that monoubiquitination of DTM-embedded PEX5 serves no purpose other than preparing the receptor for the export step, as it is necessary neither for the docking/insertion steps of PEX5 into the DTM nor for cargo protein translocation and release into the peroxisomal matrix. (196, 198). PEX5 mutant proteins that cannot be monoubiquitinated are not

substrates for the REM and accumulate at the DTM. Likewise, monoubiquitination of PEX5 in *in vitro* import assays using a GST-ubiquitin fusion protein leads to the same outcome (211). Altogether, these findings suggest that it is not the covalent modification of PEX5 *per se* that prepares the receptor for the export step (*e.g.*, by inducing a conformational alteration of PEX5), but rather that the ubiquitin moiety in the DTM-embedded Ub-PEX5 conjugate provides a context-specific "handle" for the REM.

How the REM peroxins, PEX1 and PEX6, recognize DTM-embedded Ub-PEX5 remains largely unknown. Nevertheless, recent data suggest that the interaction between Ub-PEX5 and the REM may not be direct (232). Indeed, using a mammalian *in vitro* import/export assay, Fujiki and co-workers found a cytosolic protein that stimulated export of PEX5 from the DTM. The protein was identified as AWP1, an ubiquitin-binding protein best known for its participation in the NF-κB signaling pathway (233). Further biochemical characterization of this protein led the authors to propose that AWP1 mediates the interaction between monoubiquitinated PEX5 and the REM (232).

***PEX5 deubiquitination***

Export of monoubiquitinated PEX5 from the DTM can be easily observed using a mammalian peroxisomal *in vitro* assay, particularly if the export reaction is made in the presence of a general deubiquitinase (DUB) inhibitor (*e.g.*, ubiquitin aldehyde) (230). In contrast, all attempts to detect the mammalian or yeast Ub-PEX5 thioester conjugate in cytosolic fractions obtained from cells/organs yielded negative results; Ub-PEX5 could only be detected in organelle fractions (212, 230). Apparently, *in vivo* Ub-PEX5 is deubiquitinated very rapidly after export from the DTM. Recently, UBP15 in *Saccharomyces cerevisiae* and USP9X in mammals have been identified as the deubiquitinating enzymes (DUBs) acting on Ub-PEX5. Interestingly, however, knockout and knockdown of *UBP15* and *USP9X* genes, respectively, did not result in the cytosolic accumulation of Ub-PEX5 (234, 235) suggesting there are other ways to deubiquitinate PEX5. These may include other less specific/active DUBs (234, 235) or, as proposed previously, even a non-enzymatic mechanism because the thioester bond linking ubiquitin to PEX5 is much more labile than the typical isopeptide bond found in most ubiquitin conjugates. Indeed, soluble Ub-PEX5 (but not DTM-embedded Ub-PEX5) is easily

disrupted in the presence of 5 mM glutathione (a physiological concentration) displaying a half-life of just 2.3 min (230). Ultimately, deubiquitination of PEX5 frees the receptor to undergo a new round of peroxisomal matrix protein import.

### 3.2.2. Polyubiquitination of PEX5

Monoubiquitination of PEX5 is not the only type of ubiquitination occurring at the DTM. In yeast mutant strains lacking PIM components that act at late steps of the pathway (*i.e.*, PEX5 monoubiquitination and its ATP-dependent dislocation from the DTM), small amounts of polyubiquitinated PEX5 are detected in peroxisomes (236–238). Furthermore, the steady-state levels of PEX5 are diminished in some of these mutant strains (223) suggesting that this polyubiquitination event targets PEX5 for proteasomal degradation (see Figure 2). A similar decrease in the steady-state levels of PEX5 in human cell lines from some patients with Peroxisome Biogenesis Disorders was also reported (169, 239). Further characterization of this phenomenon in yeasts revealed that polyubiquitination of PEX5 is mediated not by the peroxisome-dedicated E2 PEX4, but by the multipurpose E2s Ubc1/Ubc4/Ubc5 (236–238). Polyubiquitination of PEX5 does not seem to occur at the conserved cysteine residue; rather, one or two lysines located near the conserved cysteine have been identified as the ubiquitination sites (212, 240). Although polyubiquitination of PEX5 has not yet been detected in wild-type strains, and substitution of those two PEX5 lysines by arginines has no phenotypic effects (215), it is possible, nevertheless, that this alternative way to remove PEX5 from the DTM is important whenever the normal recycling mechanism cannot be used. Polyubiquitination events have also been reported for PEX18 and PEX20, targeting these proteins for proteasome degradation (213, 214, 241). Interestingly, a recent study suggested that dysfunctional mammalian PEX7 might undergo a similar quality control mechanism in a process mediated by the Cullin4A-RING ubiquitin E3 ligase (242).

## II- AIMS

## AIMS

Our current knowledge on the pathway followed by the receptor PEX5 during the PTS1 protein import process is reasonably detailed (16, 156, 243, 244). In contrast, our knowledge on the pathway followed by PEX7 during the PTS2 protein import is still incomplete. Actually, for mammalian PEX7, besides a recent report showing that the protein associates with peroxisomes and acquires a protease-protected status in a cytosolic ATP-independent manner not much else is presently known (245). Interestingly, that study also proposed that, contrary to PEX7, the translocation of the PTS2 protein itself would require ATP, a conclusion that is probably incorrect, as discussed in detail in a previous work from our laboratory (198). Indeed, we found that the translocation of a PTS2 protein across the peroxisomal membrane occurs upstream of PEX5 monoubiquitination, *i.e.* independently of cytosolic ATP, a finding that was later extended to PTS1 proteins (196).

Data on PEX7 from yeasts are somewhat more abundant, but not necessarily more clear. For instance, it has been suggested that yeast PEX7 interacts first with the PTS2 cargo protein and subsequently with a member of the PEX20 family (PEX20, PEX18 or PEX21); this cytosolic trimeric complex then interacts with the DTM, leading to the translocation of the cargo protein into the matrix of the organelle (246). Such pathway would suggest that PEX7 reaches the peroxisome in a cargo-dependent manner, as is in fact the case for mammalian PEX5 working in the PTS1 protein import pathway (202). Intriguingly, however, PEX7 can also be found in peroxisomes in strains lacking PEX20 and that, therefore, do not import PTS2 proteins (208).

There are also some data on the intraperoxisomal pathway followed by yeast PEX7. According to Lazarow and co-workers this protein is completely translocated across the peroxisomal membrane during its normal protein transport cycle (186). However, as stated above, these organisms use a member of the PEX20 family, and not PEX5, to transport PEX7-PTS2 cargo protein complexes to the peroxisome. Additionally, contrary to what was described for mammalian PEX7, translocation of the yeast peroxin across the peroxisomal membrane was recently proposed to occur downstream of the ubiquitination of PEX18 and therefore dependent on ATP hydrolysis (213).

Clearly, these conflicting data call for additional work. Therefore, in an effort to characterize the properties of PEX7 and PEX5 working on the PTS2 import pathway, we have optimized a previously described *in vitro* import system that was used to characterize the translocation step of thiolase. One of the first questions we wanted to address was how mammalian PEX7 is targeted to peroxisomes. Is this process PTS2-protein dependent? Does PEX7 translocate the peroxisomal membrane completely or is it retained at the DTM, similarly to PEX5 functioning in the PTS1 import pathway? What are the energetic requirements of the PEX7-mediated import pathway? Additionally, we also wanted to characterize the PEX7 recycling step. Does PEX7 undergo any posttranslational modification (*e.g.,* ubiquitination)? Is its export ATP-dependent? Could the processing of PTS2 proteins be the triggering step for cargo release or required for the export of PEX7? Finally, is PEX7 exported together with the receptor PEX5?

# III- EXPERIMENTAL PROCEDURES

**Plasmids**

pGEM4-PEX7- The cDNA coding for the full-length human PEX7 was obtained by PCR amplification using the plasmid SC119985 (OriGene) as template and the primers 5′.GCCTCTAGAGCCACCATGAGTGCGGTGTGCGGTGGA.3′ and 5′.GCGCGGTACCTCAAGCAGGAATAGTAAGAC.3′. The amplified fragment was cloned into the XbaI and the KpnI sites of pGEM4® (Promega).

pGEM4-PEX7(L70W)- A plasmid encoding PEX7 possessing a tryptophan instead of a leucine at position 70 (PEX7(L70W)) was obtained with the QuikChange® site-directed mutagenesis kit (Stratagene) using pGEM4-PEX7 as template and the primers 5′.GGAATGATGGTTGGTTTGATGTGACTTGG.3′ and 5′.CCAAGTCACAT CAAACCAACCATCATTCC.3′.

pGEM4-preL4RPEX7- A plasmid encoding a PEX7 protein possessing at its N terminus the peptide MAQRRQVVLGHLRGPADSGWMPQAAPCLSGASR (preL4R-PEX7) was constructed as follows. The plasmid SC119985 was used as template in a PCR reaction with the primers 5′.GCCTCTAGAATGAGTGCG GTGTGCGGTGGA.3′ and 5′.GCGCGGTACCTCAAGCAGGAATAGTAAGAC.3′. The obtained DNA fragment was inserted into XbaI/KpnI-digested pGEM4® (Promega). This plasmid was then digested with SphI and XbaI and ligated to the pre-annealed primers 5′.CCACCATGGCGCAGAGGCGGCAGGTAGTGCTGGGCCACCTGAG GGGTCCGGCCGATTCCGGCTGGATGCCGCAGGCCGCGCCTTGCCTGAGCGGTGCCT.3′ and 5′.CTAGAGGCACCGCTCAGGCAAGGCGCGGCCTGCGGCATCCAGCCGGAATCG GCCGGACCCCTCAGGTGGCCCAGCACTACCTGCCGCCTCTGCGCCATGGTGGCATG.3′. The peptide preceding PEX7 in the pre-L4R-PEX7 fusion protein contains amino acid residues 2-30 of human pre-thiolase in which leucine 4 was replaced by an arginine (numbering of full-length human pre-thiolase (247)). This peptide still contains the cleavage site for the matrix processing peptidase, but the L4R mutation abolishes its PTS2 function (248).

pGEM4-pre-Thiolase- The plasmid encoding full-length human thiolase precursor was described elsewhere (198).

pGEM4-preL4R-Thiolase- A plasmid coding for pre-thiolase possessing the L4R mutation was obtained with the QuickChange® site-directed Mutagenesis Kit (Stratagene), using pGEM4-pre-Thiolase as template and the primers 5′.ATGCAGAGGCGGCAGGTAGTGCTGGG.3′and 5′.CCCAGCACTACCTGCCGCCT CTGCAT.3′.

pGEM4-PEX5L- The plasmid encoding the large isoform of human PEX5 (PEX5L) was described before (200).

pET28-ΔC1PEX5L(C11A)- The plasmid encoding amino acid residues 1-324 of PEX5L possessing an alanine at position 11 (ΔC1PEX5L(C11A)) was obtained with the QuikChange® site-directed mutagenesis kit (Stratagene), using pET28-ΔC1PEX5L as template (147) and primers described elsewhere (230).

pET28-PEX7- The cDNA coding for histidine-tagged PEX7 ($His_6$PEX7) was obtained by PCR amplification using the plasmid SC119985 (OriGene) as template and the primers 5′.GTATGAGCCATATGAGTGCGGTGTGCGGTGGAG.3′ and 5′.GGCCGCGGAATTCTCAAGCAGGAATAGTAAGAC.3′. The amplified fragment was cloned into the NdeI and the EcoRI sites of pET-28a (Novagen).

pQE31-PHYH- The plasmid encoding the precursor form of human Phytanoyl-CoA hydroxylase (p-PHYH) was described in (249).

pQE31-m-PHYH- The cDNA encoding the mature form of this protein (m-PHYH) was obtained by PCR amplification of the plasmid pQE31-PHYH using the primers 5′.GGCGCGGTACCATCAGGGACTATTTCCTCTGCC.3′ and 5′.GGCGCAAGCTTTCA AAGATTGGTTCTTTCTCC.3′ and cloned into the KpnI and HindIII sites of pQE31 (Qiagen).

pGEM4-PHYH- The plasmid encoding the precursor form of human Phytanoyl-CoA hydroxylase was obtained by PCR amplification of the plasmid pQE31-PHYH using the primers 5′.GACAAAGCTTGCCACCATGGAGCAGCTTCG.3′ and 5′.GGGCGCGAATTCTCAAAGATTGGTTCTTTCTC.3′ and cloned into the HindIII and EcoRI sites of pGEM4® (Promega).

pQE31-PHYH(Δ29-30) and pGEM4-PHYH(Δ29-30)- Plasmids encoding a mutant version of p-PHYH where the residues Pro29 and Thr30 were deleted. These were obtained with the QuikChange® site-directed mutagenesis kit (Stratagene), the primers 5′.GGGGCTGTCGTAGCTCATTCAGGGACTATTTCCTCTGC.3′ and 5′.GCAGAGGAAATAGTCCCTGAATGAGCTACGACAGCCCC.3′ and the plasmids pQE31-PHYH and pGEM4-PHYH as the respective templates.

**Recombinant Proteins**

The recombinant large isoform of human PEX5 (PEX5L) (168), PEX5L containing the missense mutation N526K (PEX5L(N526K)) (209), proteins comprising amino acid residues 1-324 or 315-639 of PEX5L (ΔC1PEX5L and TPRs, respectively) and ΔC1PEX5L containing the missense mutation C11A (ΔC1PEX5L(C11A)) (147, 171), a protein comprising the first 287 amino acid residues of the small isoform of human PEX5 (ΔC1PEX5S) (198), the GST-ubiquitin fusion protein (GST-Ub) (211), the precursor of human Phytanoyl-CoA hydroxylase (p-PHYH), its mature form (m-PHYH) and the mutant PHYH(Δ29-30) (249), human PEX19 (128) and a protein comprising the first 80 amino acid residues of human PEX14 (NDPEX14) (171), were obtained as described previously. Histidine-tagged PEX7 was expressed in the BL21(DE3) strain of *Escherichia coli* and obtained as inclusion bodies. The fusion protein was purified by HIS-Select™ nickel affinity gel (Sigma) under denaturing conditions (6 M Guanidine Hydrochloride) and concentrated by trichloroacetic acid precipitation.

**Preparation of postnuclear supernatants (PNS) from rat and mouse liver**

Rat liver PNS fractions for *in vitro* assays were prepared from male Wistar rats with 6 to 8 weeks of age. The animal was fasted overnight, made unconscious in a $CO_2$ chamber and euthanized by cervical dislocation. The liver was quickly homogenized in ice-cold SEM buffer (0.25 M sucrose, 20 mM MOPS-KOH, pH 7.2, 1 mM EDTA-NaOH, pH 8.0) supplemented with 2 µg/ml N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide (E-64). After centrifuging the

homogenates twice at 600 x g for 10 min at 4 ºC (SS-34 rotor in a RC5B Sorvall® centrifuge), the resulting supernatant was aliquoted, frozen in liquid nitrogen and stored at -70 ºC. Postnuclear supernatants from *PEX7* knockout male mice with approximately 12 weeks of age were prepared exactly as described above. Protein content of PNS fractions was determined using the Bradford method of protein quantitation.

**In Vitro Import/Export Reactions**

In a typical import reaction (100 µl final volume), $^{35}$S-labeled proteins (1-2 µl of the rabbit reticulocyte lysates; see below) were diluted to 10 µl with import buffer (20 mM MOPS-KOH, pH 7.4, 0.25 M sucrose, 50 mM KCl, 3 mM $MgCl_2$, 20 µM methionine, 2 µg/ml E-64, 2 mM reduced glutathione, final concentrations) and added to 500 µg of liver PNS that had been primed for import (5 min incubation at 37 ºC in import buffer containing 0.3 mM ATP; see (198, 203) for details). Reactions were incubated for 30 min at 37 °C, unless otherwise stated. ATP or AMP-PNP were used at 3 mM, final concentration. NTP depletion from both PNS and reticulocyte lysates using apyrase (Grade VII, Sigma) was done exactly as described (196). Where indicated, import reactions were supplemented with recombinant PEX5 proteins (PEX5L, PEX5L(N526K), ΔC1PEX5L, ΔC1PEX5L(C11A) or ΔC1PEX5S; 30 nM final concentrations), GST-Ub or bovine ubiquitin (10 µM), and recombinant p-PHYH, m-PHYH or p-PHYH(Δ29-30) (140 nM, final concentration). After import, reactions were treated with pronase (500 µg/ml final concentration) for 45 min on ice and processed for SDS-PAGE/autoradiography exactly as described before (198). In some experiments, organelles were resuspended in import buffer and subjected to pronase digestion in the presence or absence of 1% (w/v) Triton X-100.

In the *in vitro* export assays, radiolabeled proteins were first subjected to an import assay for 15 min. Further import was then stopped either by adding recombinant NDPEX14 to the reaction (30 µM final concentration), or by isolating the organelles by centrifugation and resuspending them in import buffer. In earlier experiments, cytosolic proteins derived from 500 µg of liver PNS were also added.

The organelle suspensions were then incubated at 37 ºC in the presence of either 5 mM ATP or AMP-PNP.

For the PTS2-only *in vitro* import/export experiments, PNS were pre-incubated with 1 µM recombinant TPRs for 10 min on ice, before starting the import assays. This recombinant protein, corresponding to the C-terminal half of PEX5, comprises its PTS1-binding domain and is used here to sequester endogenous PTS1-containing proteins (174, 202, 209). Also, the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-PEX5L (2 µl each) were pre-incubated with recombinant p-PHYH (20 min at 23 ºC in 10 µl of import buffer) to favor formation of the trimeric PEX5L-PEX7-PTS2 complex. The export incubation was carried out as described above, but in the presence of 1 µM TPRs and 10 µM NDPEX14.

Any variations in protocol (duration of the import assays, concentrations of recombinants, incubation conditions) are explicitly referred to in the corresponding figure legend or when describing the experiment in the results section.

**Fractionation of organelles by sonication and centrifugation**

Pronase-treated organelles from an import reaction or rat liver purified peroxisomes were resuspended in 20 mM MOPS-KOH, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 2 mM DTT, 0.1 mg/ml phenylmethanesulfonylfluoride, 1:500 (v/v) mammalian protease inhibitors mixture (Sigma) and disrupted by sonication using a SONOPULS HD2200-BANDELIN equipped with a MS 73 microtip. The sonication conditions used (40% duty cycle, 10% output power for just 25 s) were established as the mildest ones resulting in a quantitative extraction of catalase from peroxisomes. Membrane and matrix components were separated by centrifugation at 100,000 g for 60 min at 4 ºC.

**Miscellaneous**

All $^{35}$S-labeled proteins were synthesized using the TNT® T7 quick coupled transcription/translation kit (Promega) in the presence of [$^{35}$S]methionine (specific activity >1000 Ci/mmol; PerkinElmer Life Sciences). The antibody directed against

human PEX7 was produced in rabbits using recombinant histidine-tagged PEX7. The antibody directed to PEX13 was described elsewhere (122) and the one against catalase was purchased from Research Diagnostics, Inc. (catalogue number RDI-CATALASEabr). All antibodies were detected using goat alkaline phosphatase-conjugated anti-rabbit antibodies (A9919; Sigma).

Densitometric analyses of autoradiography films were performed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011).

The number or DTMs per peroxisomes was estimated as follows: 1 g of rat liver has the equivalent of 260 mg of total protein and an estimated 5.9 $x10^{10}$ peroxisomes (67, 250). Approximately 2.5% of the total proteins corresponds to peroxisomal protein (67) (2.5% x 260 mg = 6.5 mg of peroxisomal protein/ g of liver). PEX14 was estimated at 0.25% of all peroxisomal protein (142) (0.25% x 6.5 mg = 16.25 µg of PEX14/ g of liver). With a MW of ~41 kDa, 16.26 µg of PEX14 corresponds to 4.0 $x10^{-10}$ moles or 2.4 $x10^{14}$ molecules of PEX14/ g of liver. Dividing by the number of peroxisomes, we obtain a number of 4,000 molecules of PEX14 molecules per peroxisome. Considering a theoretical ratio of 7 molecules of PEX14 per DTM (a molar ratio of 1:7 was determined for PEX5 and PEX14 at the peroxisomal membrane (142)), we estimate that there are approximately 500 DTMs per peroxisome.

# IV- RESULTS

## 1. PEX7 reaches the peroxisome in a PEX5L- and PTS2-dependent manner.

Protein *in vitro* import systems have been used with great success to understand how peroxisomal matrix proteins are imported into peroxisomes. Essentially, an *in vitro* import reaction consists in incubating a $^{35}$S-labeled reporter protein with an organelle suspension under physiological conditions. After incubation, large amounts of a non-specific protease are added to the reaction to degrade non-imported $^{35}$S-labeled protein while the fraction that was imported into the organelles is preserved. We have recently described an improved *in vitro* system to characterize the peroxisomal import mechanism of pre-thiolase, a PTS2-containing protein (198). The system relies on a rat liver postnuclear supernatant as a source of peroxisomes and cytosolic components, supplemented with either recombinant ΔC1PEX5L (amino acid residues 1-324 of PEX5L) or PEX5L(N526K) (PEX5L possessing a lysine at position 526 instead of an asparagine). These two PEX5 proteins contain an intact PEX7-binding domain as well as all the other elements required for a productive interaction with the peroxisomal protein import machinery (145, 209), and thus they are still competent in the PTS2-mediated import pathway. However, they do not bind efficiently PTS1 proteins (145, 148, 199, 251), an advantage when studying the PTS2-mediated import pathway (see below). In this work we used this improved system to characterize the pathway followed by PEX7 during the protein transport cycle it promotes.

Figure 4 shows the results of *in vitro* import assays performed with both $^{35}$S-labeled PEX7 and $^{35}$S-pre-thiolase. In the absence of ΔC1PEX5L, or in the presence of ΔC1PEX5S (a protein almost identical to ΔC1PEX5L that lacks the PEX7-binding domain; see Introduction), only a small fraction of protease-protected (imported) thiolase was observed in organelle pellets, as expected (198), and the same is true for $^{35}$S-PEX7 (lanes 1 and 3). A 5-fold increase in the amounts of both radiolabeled proteins was observed when the import assays were supplemented with either recombinant ΔC1PEX5L or PEX5L(N526K) (lanes 2 and 5). Recombinant PEX5L also improves the import efficiencies of both pre-thiolase and PEX7 but only by a factor of ≈ 2.5 (compare lanes 1 and 4). The weaker stimulatory effect obtained with PEX5L is probably due to the fact that this protein

also interacts with endogenous PTS1-containing proteins present in the PNS, creating a competition problem at the peroxisomal DTM (see also (198)).

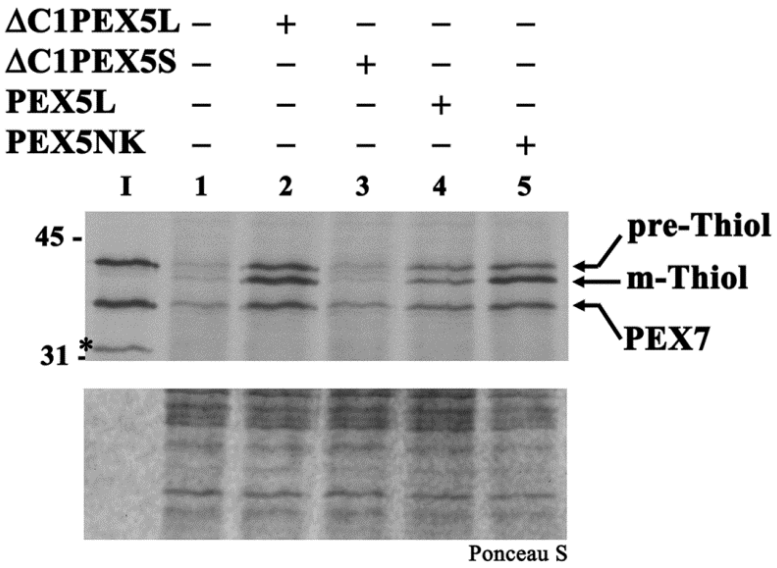


**Figure 4. $^{35}$S-labeled PEX7 acquires a protease-protected status in *in vitro* import reactions fortified with PEX5L(N526K) or ΔC1PEX5L.**

A rat liver PNS was incubated with $^{35}$S-labeled PEX7 and $^{35}$S-pre-thiolase in import buffer containing ATP in the absence (lane 1) or presence of recombinant ΔC1PEX5L (lane 2), ΔC1PEX5S (lane 3), PEX5L (lane 4), or PEX5L(N526K) (lane 5). Pronase-treated organelles were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. Lane I, 10% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-pre-thiolase used in each reaction. Autoradiographs (upper panels) and corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa. The asterisk marks a radiolabeled band occasionally produced by the in vitro translation kit in an unspecific manner. pre-Thiol and m-Thiol refer to the precursor and mature forms of thiolase, respectively.

The *in vitro* import yields of $^{35}$S-PEX7 obtained in the presence of ΔC1PEX5L can be further improved by a factor of 2 when a recombinant PTS2 protein, pre-phytanoyl-CoA 2-hydroxylase (p-PHYH), is added to the assay (Figure 5A, compare lanes 3 and 4). The stimulatory effect of p-PHYH on PEX7 import contrasts with its inhibitory effect on pre-thiolase import (Figure 5A, compare lanes 3 and 4). Recombinant phytanoyl-CoA 2-hydroxylase lacking the PTS2 (m-PHYH) has no such effects (Figure 5B, compare lane 1 with 2 and 3, respectively). These findings strongly suggest that the $^{35}$S-PEX7 protein used in these experiments is truly functioning in the PTS2-mediated protein import pathway.

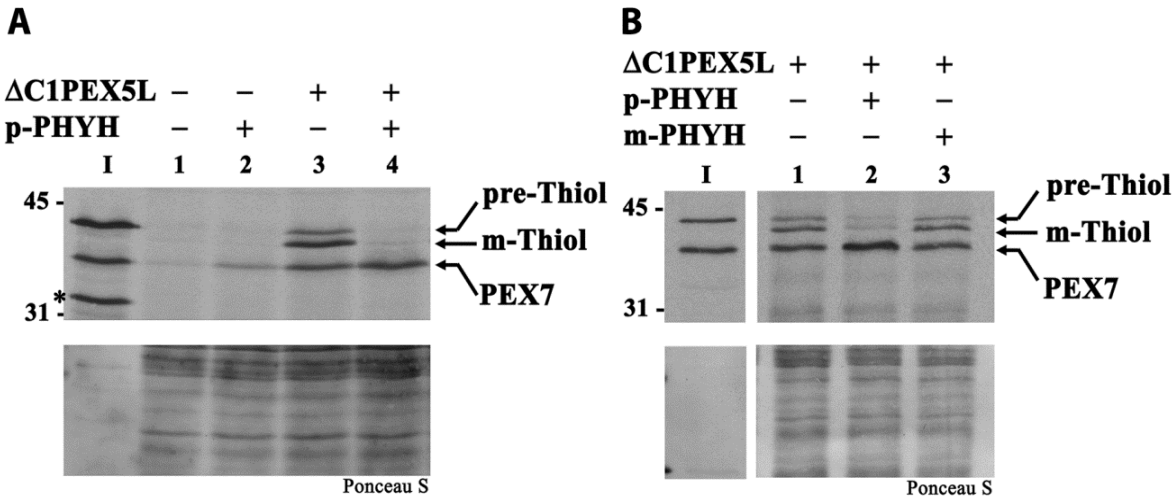


**Figure 5. Import of $^{35}$S-PEX7 is stimulated in the presence of a recombinant PTS2-containing protein.**

**A**, A rat liver PNS was incubated with $^{35}$S-labeled PEX7 and $^{35}$S-pre-thiolase in import buffer containing ATP in the absence (lanes 1 and 2) or presence of recombinant ΔC1PEX5L (lanes 3 and 4). Import reactions were supplemented (lanes 2, 4) or not (lanes 1, 3) with recombinant p-PHYH. Pronase-treated organelles were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. **B**, ΔC1PEX5L-supplemented *in vitro* import assays of $^{35}$S-PEX7 and $^{35}$S-pre-thiolase in the absence (lane 1) and in the presence of either p-PHYH or m-PHYH (lanes 2 and 3, respectively). Samples were processed as in A. Lanes I in A and B, 10% and 5% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-pre-thiolase used in each reaction, respectively. Autoradiographs (upper panels) and corresponding Ponceau S-stained membranes (lower panels) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa. The asterisk in A marks a radiolabeled band occasionally produced by the *in vitro* translation kit in an unspecific manner.

Further data corroborating this conclusion were obtained when a PNS from *PEX7* knockout mice (252) was used in import assays. As shown in Figure 6, an assay using PNS from these mice supplemented with ΔC1PEX5L (and 2 μl of a mock-translated reticulocyte lysate) failed to reveal import of pre-thiolase (lane 1). In contrast, addition of just 2 μl of the lysate containing $^{35}$S-PEX7 was sufficient to promote import and partial processing of pre-thiolase (Figure 6, lane 2). A non-functional PEX7 protein harboring a mutation previously described in a patient with Rhizomelic Chondrodysplasia Punctata Type 1 (PEX7(L70W); (253)) was used as a negative control and, as expected, was not competent in this assay (Figure 6, lane 3).

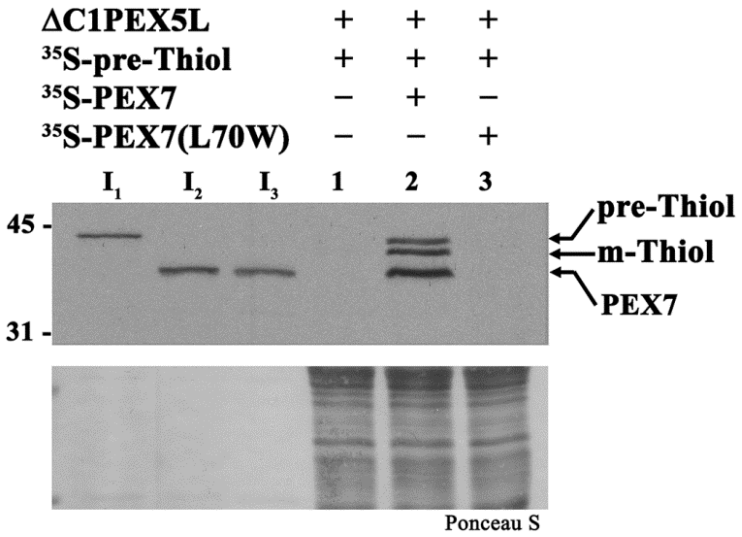


**Figure 6. PEX7 but not PEX7(L70W) promotes import of $^{35}$S-pre-thiolase into peroxisomes from *PEX7* knockout mice.**

PNS from *PEX7* knockout mouse liver was used in import assays with $^{35}$S-pre-thiolase in the presence of either a mock-translated reticulocyte lysate (lane 1) or lysates containing $^{35}$S-PEX7 (lane 2) or $^{35}$S-PEX7(L70W) (lane 3). Samples were processed as in Figure 4. Lanes $I_1$, $I_2$ and $I_3$, 5% of the reticulocyte lysates containing $^{35}$S-pre-thiolase, $^{35}$S-PEX7 and $^{35}$S-PEX7(L70W) used in the reactions, respectively. Autoradiograph (upper panel) and corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

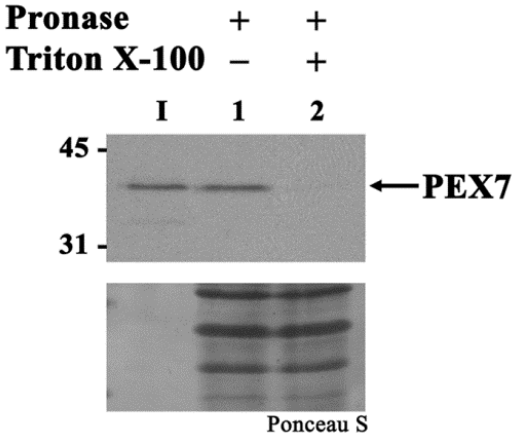


**Figure 7. Imported $^{35}$S-PEX7 is protected from proteases by a lipid membrane.**

Organelles from an import assay made in the presence of recombinant ΔC1PEX5L and p-PHYH were isolated by centrifugation, resuspended in import buffer and subjected to pronase digestion in the absence (lane 1) or presence (lane 2) of 1% (w/v) Triton X-100. Lane I, 5% of the reticulocyte lysate containing $^{35}$S-PEX7. Autoradiograph (upper panel) and corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

It is important to note that the resistance of *in vitro* imported PEX7 to the protease does not reflect an intrinsic property of PEX7 because no radiolabeled protein is detected when the protease treatment is made in the presence of Triton X-100, a mild detergent that solubilizes biological membranes (Figure 7, lane 2). Taken together, the experiments described above strongly suggest that *in vitro* synthesized PEX7 reaches the peroxisome in a PEX5L- and PTS2-dependent manner where it acquires a protease-protected status.

**2. The energetic requirements of PEX7 import.**

It was previously shown that: 1) PEX5L becomes inserted into the DTM in a cytosolic ATP-independent process (199, 200, 203), and 2) translocation of pre-thiolase across the DTM into the peroxisomal matrix occurs upstream of the first cytosolic ATP-dependent step, *i.e.* before monoubiquitination of PEX5 (198). Not surprisingly, we found that the energetic requirements of PEX7 import are identical, as was in fact also reported by others (245). As shown in Figure 8A, neither supplementation of import reactions with AMP-PNP (a non-hydrolyzable ATP analog; (235, 254)), nor pre-treatment of the $^{35}$S-PEX7 protein and PNS with apyrase (an enzyme that hydrolyzes ATP and other NTPs; (255)) blocked PEX7 import (compare lane 1 with lanes 2 and 3, respectively).

Interestingly, although export of peroxisomal PEX7 is ATP-dependent (see section IV-4 below), the levels of peroxisomal PEX7 observed under the different energetic conditions are identical. This finding suggests that export of PEX7 from the peroxisome becomes a rate-limiting step in this optimized *in vitro* import system.

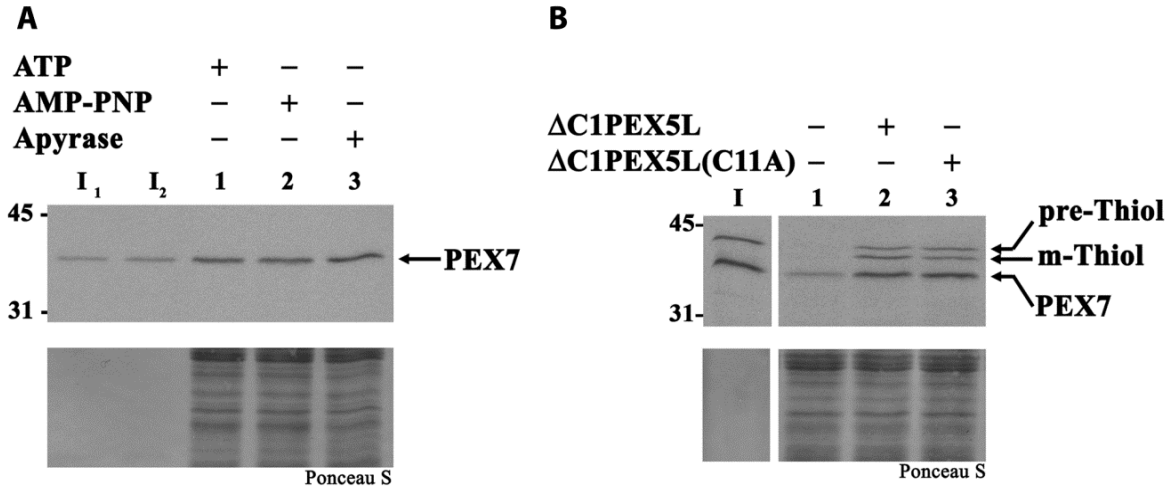


**Figure 8. The energetics of PEX7 import.**

**A**, A primed rat liver PNS fraction (see Experimental Procedures) was incubated with $^{35}$S-PEX7 for 7 min in import buffer containing ΔC1PEX5L and p-PHYH in the presence of either ATP (lane 1), or AMP-PNP (lane 2). An identical assay but using apyrase-treated PNS and $^{35}$S-PEX7 was also performed (lane 3). Lanes $I_1$ and $I_2$, 5% of the reticulocyte lysates containing $^{35}$S-PEX7 used in lanes 1 and 2 (- apyrase), and lane 3 (+ apyrase), respectively. **B,** A non-monoubiquitinatable form of PEX5 (ΔC1PEX5L(C11A)) is as efficient as wild-type PEX5 in targeting PEX7 and pre-thiolase to the peroxisome. Import assays with $^{35}$S-PEX7 and $^{35}$S-pre-thiolase were made in import buffer containing ATP and GST-Ub, in the absence (lane 1) or presence of recombinant ΔC1PEX5L (lane 2) or ΔC1PEX5L(C11A) (lane 3). Note that ubiquitination of ΔC1PEX5L with GST-Ub results in a species that is no longer export-competent (147, 211). Pronase-treated organelles were analyzed as described in Figure 4. Autoradiographs (upper panels) and the corresponding Ponceau S-stained membranes (lower panels) are shown. Lane I, 5% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-pre-thiolase were mixed and loaded together in the same lane.

The data in Figure 8A showing that PEX7 import is not blocked in assays containing apyrase, a condition previously shown to block PEX5 monoubiquitination (196, 198), also suggests that import of PEX7, like import of pre-thiolase, occurs upstream of PEX5L-monoubiquitination. Additional data supporting this conclusion are presented in Figure 8B. Identical amounts of protease-protected $^{35}$S-PEX7 and thiolase were obtained in import reactions supplemented with either ΔC1PEX5L or ΔC1PEX5L(C11A), a mutant protein that possesses an alanine at position 11. The substitution of cysteine 11 by an alanine results in a PEX5 protein that can still enter the DTM but that is no longer monoubiquitinated (230).

### 3. The N terminus of peroxisomal PEX7 is exposed into the organelle matrix.

The fact that peroxisomal $^{35}$S-PEX7 is resistant to exogenously added proteases suggests that PEX7 exposes no major domains into the cytosol but provides no clues on how deep in peroxisomes it reaches. To address this issue we adapted a strategy previously used by others to show that a portion of the polypeptide chain of peroxisomal PEX5 reaches the peroxisomal matrix (248). Specifically, we synthesized a PEX7 protein having at its N terminus a cleavable, but otherwise non-functional, mutant version of thiolase pre-sequence and asked whether this PEX7 protein (hereafter referred to as preL4R-PEX7) could be cleaved in our *in vitro* import assays. A control experiment with a pre-thiolase carrying the same mutation (L4R) confirmed that this mutant pre-sequence is not functional in our *in vitro* assays (Figure 9A and (256)).

As shown in Figure 9B, preL4R-PEX7 subjected to *in vitro* import assays not only acquired a protease-resistant status in a PEX5L- and PTS2-dependent manner, but was also converted into a 2-3 kDa shorter protein. Furthermore, preL4R-PEX7, like PEX7, was also able to restore import of pre-thiolase in PNS from the *PEX7* knockout mice (Figure 9C). Processing of preL4R-PEX7 requires its passage through the peroxisome because nearly no processed PEX7 could be detected when the import assays were performed in the presence of a recombinant protein comprising the PEX5-binding domain of PEX14 ((257); Figure 10, compare lanes 1 and 5 with lanes 3 and 7, respectively). As shown before, this recombinant protein completely blocks the PEX5-mediated protein import pathway (196). Interestingly, when the protease treatment was omitted, cleaved PEX7 was also detected in the supernatant of the import assays but only under import-permissive conditions (Figure 10, compare lanes 2 and 4) suggesting that our *in vitro* system can also be used to study PEX7 export.

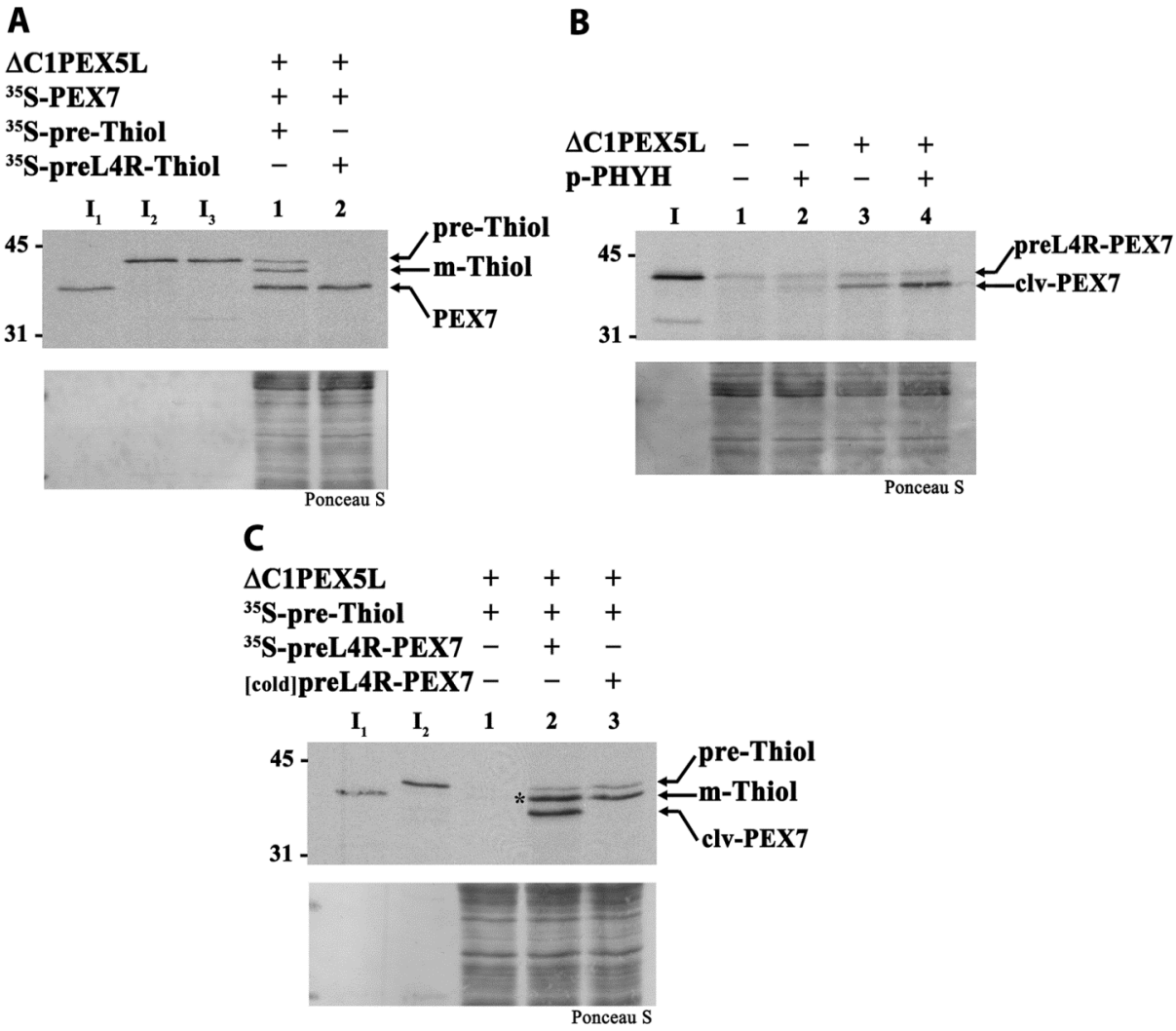


**Figure 9. PEX7 becomes transiently exposed to the organelle matrix during the PTS2-mediated protein import pathway.**

**A**, $^{35}$S-pre-thiolase containing an arginine instead of a leucine at position 4 (preL4R-Thiol; lane 2) is not imported *in vitro*. Lanes $I_1$, $I_2$ and $I_3$, 5% of the reticulocyte lysates containing $^{35}$S-PEX7, $^{35}$S-pre-thiolase and $^{35}$S-preL4R-thiolase, respectively. **B,** $^{35}$S-preL4R-PEX7 was subjected to import assays in the absence (lane 1) or presence of the indicated recombinant proteins (lanes 2-4). Pronase-treated organelles were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. clv-PEX7, cleaved $^{35}$S-preL4R-PEX7. **C**, $^{35}$S-preL4R-PEX7 promotes import of $^{35}$S-pre-thiolase into peroxisomes from *PEX7* knockout mice. PNS from *PEX7* knockout mice was used in import assays with $^{35}$S-pre-thiolase in the presence of either a mock-translated reticulocyte lysate (lane 1) or a lysate containing $^{35}$S-preL4R-PEX7 (lane 2). Import and processing of $^{35}$S-pre-thiolase is best seen in import assays using unlabeled/cold preL4R-PEX7 (lane 3) due to the fact that mature thiolase co-migrates with uncleaved pre-L4R-PEX7 (lane 2, asterisk). Lanes $I_1$ and $I_2$, 5% of the reticulocyte lysates containing $^{35}$S-preL4R-PEX7 and $^{35}$S-pre-thiolase used. Autoradiographs (upper panels) and corresponding Ponceau S-stained membranes (lower panels) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

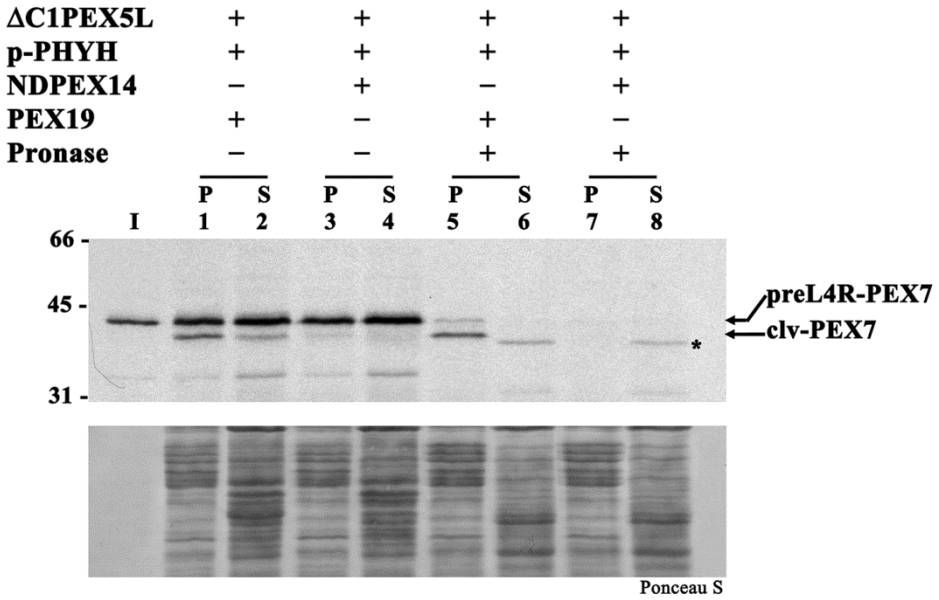


**Figure 10. Processing of $^{35}$S-preL4R-PEX7 in import assays occurs only under import-permissive conditions.**

$^{35}$S-preL4R-PEX7 was subjected to import assays in the presence of the indicated recombinant proteins. At the end of the incubation the samples were halved and treated or not with pronase, as indicated. The import reactions were then centrifuged to obtain organelle pellets (P) and supernatants (S). Total pellets (derived from 500 µg of PNS) and ¼ of the corresponding supernatants were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The asterisk indicates a soluble minor preL4R-PEX7-derived fragment displaying some resistance to pronase. PEX19, a protein involved in another aspect of peroxisome biogenesis (128), was used in these assays as a negative control for NDPEX14. . Lanes I, 5% of the reticulocyte lysates containing $^{35}$S-preL4R-PEX7 used in these assays. Autoradiograph (upper panel) and corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

Finally, and in agreement with the data shown in Figure 8B, cleavage of preL4R-PEX7 was also observed when its import was promoted by ΔC1PEX5L(C11A) (Figure 11). Interestingly, when this export-incompetent PEX5 species is used in these assays, almost no cleaved PEX7 is recovered in the supernatant fraction (Figure 11, compare lanes 3 and 4) suggesting that export of cleaved PEX7 is somehow dependent on PEX5 ubiquitination/export (see also below). In summary, these results indicate that at least the N terminus of PEX7 reaches a location where it can be cleaved by the protease that processes PTS2 proteins, *i.e.*, the peroxisomal matrix.

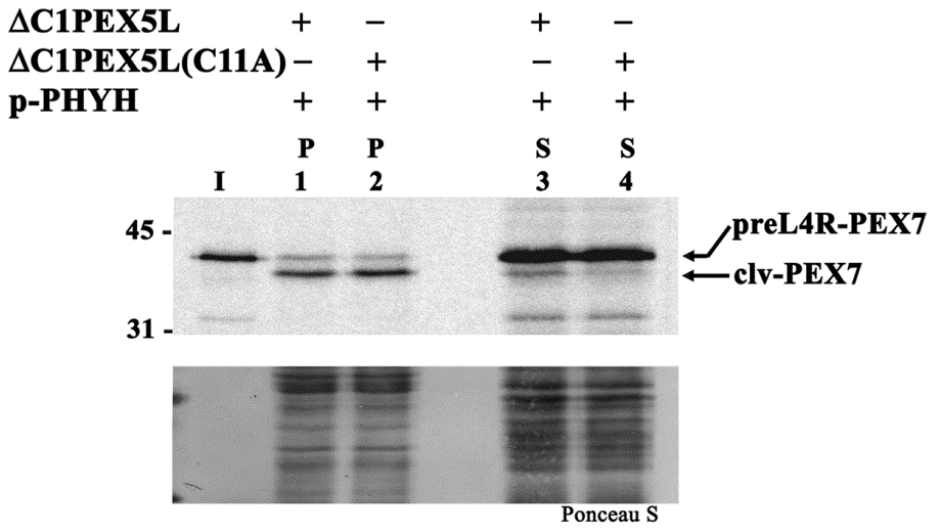


**Figure 11. Accessibility of $^{35}$S-preL4R-PEX7 to the peroxisomal matrix does not depend on PEX5 ubiquitination/ export.**
Import assays with $^{35}$S-preL4R-PEX7 were performed in the presence of ΔC1PEX5L (lanes 1 and 3) or ΔC1PEX5L(C11A) (lanes 2 and 4). Pronase-treated organelles (lanes P) and untreated supernatants (lanes S) were analyzed as in Figure 10. Lanes I, 5% of the reticulocyte lysate containing $^{35}$S-preL4R-PEX7 used in each reaction. Autoradiograph (upper panel) and corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

## 4. Export of PEX7 from the peroxisome requires export of PEX5L, but the two events are not strictly coupled.

PEX7 functions as a shuttling receptor, meaning that peroxisomal PEX7 is eventually exported back to the cytosol (186). Aiming at characterizing in detail this process we developed a two-step protocol in which $^{35}$S-PEX7 is first subjected to an import assay, and after blocking further import (see Experimental Procedures for details), the organelle suspension is then subjected to a second incubation step, the export assay. The results of one of these assays performed under standard conditions show that the amount of organelle-associated protease-protected $^{35}$S-PEX7 decreases over time with the concomitant appearance of $^{35}$S-PEX7 in the supernatant (Figure 12). Note that there is a small amount of PEX7 already in the soluble phase at 0 minutes of export. We attribute this to the release of a small amount of PEX7 that is unspecifically absorbed to the membrane when the protease-untreated organelles are resuspended.

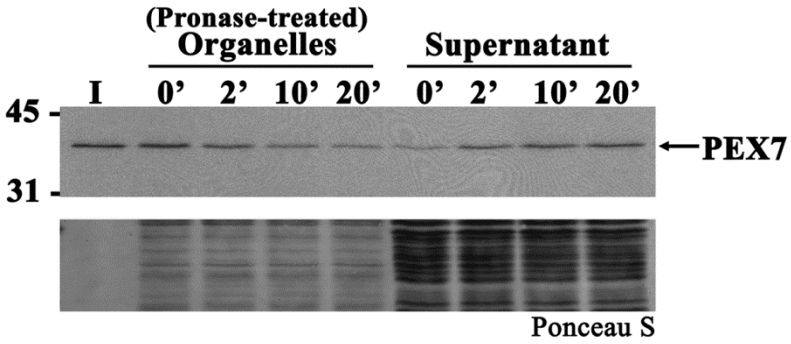


**Figure 12. *In vitro* imported PEX7 is exported back to the cytosol.**
$^{35}$S-PEX7 was imported for 15 min in the presence of p-PHYH, ΔC1PEX5L, ubiquitin and ATP. The reaction mix was then diluted with ice-cold import buffer and the organelles were isolated by centrifugation and subjected to an export assay in the presence of ATP (see Experimental Procedures for details). Aliquots were collected at the indicated time points, and one half was treated with pronase while the other was left untreated. Equivalent amounts of organelles from the pronase-treated aliquots and supernatants from the untreated aliquots (derived from 125 µg of PNS) were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. Lanes I, 5% of the reticulocyte lysates containing $^{35}$S-PEX7 used in each reaction. Autoradiograph (upper panels) and the corresponding Ponceau S-stained membrane (lower panels) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

Interestingly, experimental conditions that inhibit export of peroxisomal PEX5 back into the cytosol, also block export of PEX7. As shown in Figure 13A, almost no export of PEX7 was detected in assays made in the presence of AMP-PNP. This non-hydrolyzable ATP analogue still allows PEX5 monoubiquitination at the DTM but blocks the receptor export module (235). A similar inhibition was observed when both the import and export incubations were made in the presence of a GST-ubiquitin fusion protein (GST-Ub, Figure 13B). As shown before, ubiquitination of DTM-embedded PEX5 with this ubiquitin analogue results in a species that is no longer export-competent (211). Note that we have been unable to detect any ubiquitination of PEX7 in our *in vitro* assays (even under non-reducing conditions; data not shown) suggesting that the effect of GST-Ub on PEX7 export occurs via PEX5. In agreement with this interpretation, and with the data shown in Figure 11, when $^{35}$S-PEX7 was imported in the presence of ΔC1PEX5L(C11A) no significant export of $^{35}$S-PEX7 was detected (Figure 13C). Thus, peroxisomal PEX7 is exported back into the cytosol only when PEX5 is also exported.

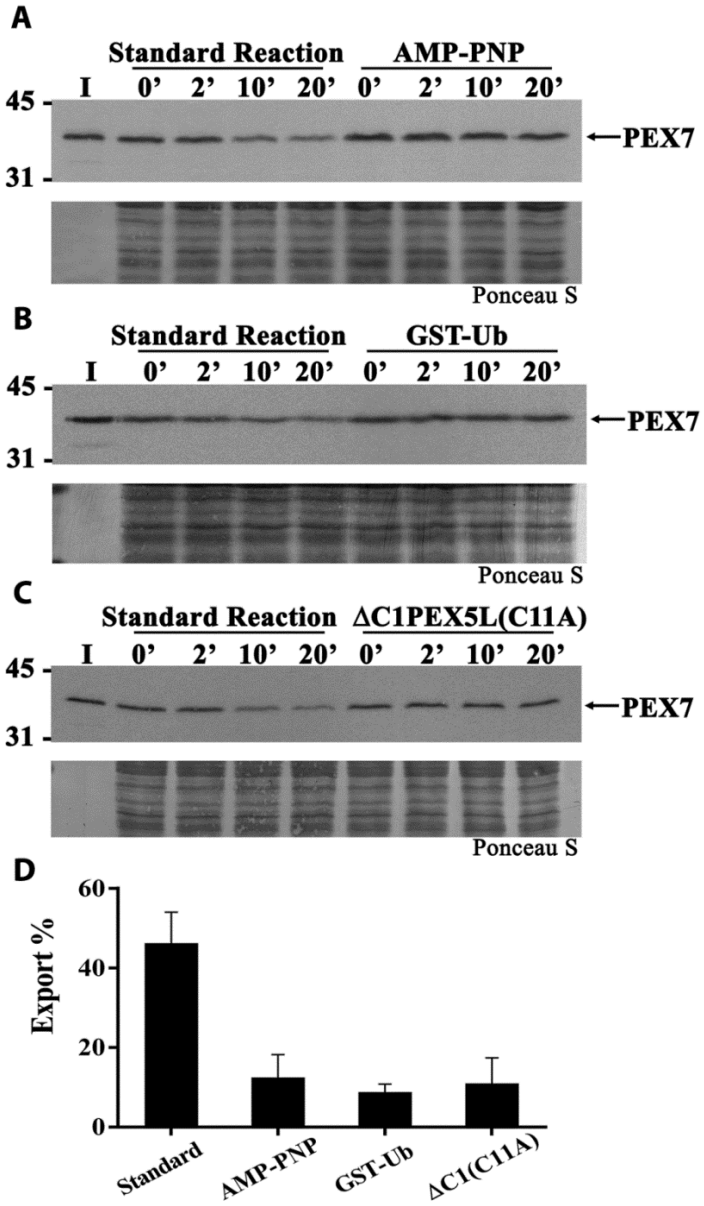


**Figure 13. PEX5 export is a requirement for PEX7 export.**

**A-C**, In "standard reactions", the ΔC1PEX5L- and p-PHYH-mediated import of $^{35}$S-PEX7 was allowed to occur at 37 ºC for 15 min in the presence of ubiquitin and ATP. At this point, import was inhibited by the addition of NDPEX14 (30 μM) and the reaction further incubated. Aliquots were taken at the indicated time points. Pronase-treated organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. Autoradiograph (upper panels) and the corresponding Ponceau S-stained membrane (lower panels) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa. PEX7 export was inhibited when ATP was replaced by AMP-PNP (**A**). Likewise, replacing ubiquitin by GST-Ub in the import step inhibits subsequent export of PEX7 (**B**). The same inhibition was observed when recombinant ΔC1PEX5L was replaced by ΔC1PEX5L(C11A) (**C**). Lanes I, 5% of the reticulocyte lysates containing $^{35}$S-PEX7 used in each assay. **D**, The bar graph shows the average percentage of PEX7 export after 20 min under the conditions described in A, B and C. Standard deviations (n≥3) are also presented.

Several hypotheses could explain this phenomenon. An obvious one would be to assume that export of PEX7 is coupled to that of PEX5. Alternatively, it might be that PEX5 arrested at the DTM simply blocks the site used by PEX7 to exit the organelle. In an attempt to clarify this issue we determined the export kinetics of both proteins. Obviously, such an experiment would only be informative if we could find conditions where PEX5 would reach the peroxisome in a PTS2-only mode. With this in mind, we performed *in vitro* assays in the presence of a recombinant protein comprising the PTS1-binding domain of PEX5 (referred to as TPRs), a strategy previously shown to efficiently block the PTS1-dependent targeting of PEX5 to the peroxisome (202, 209), and asked whether peroxisomal targeting of $^{35}$S-PEX5 could be recovered by adding $^{35}$S-PEX7 and recombinant p-PHYH to the import assays. As shown in Figure 14A, this strategy turned out to be feasible: import of $^{35}$S-PEX5L was now PEX7/PTS2-dependent. Using these experimental conditions we then employed the two-step import-export protocol described above to compare the export kinetics of $^{35}$S-PEX7 and $^{35}$S-PEX5L. Briefly, after an import step performed in presence of AMP-PNP, the organelles were isolated by centrifugation, resuspended in import buffer and subjected to an export assay. Aliquots were then withdrawn at various time points, and protease-treated organelles were analyzed by SDS-PAGE/autoradiography. As shown in Figure 14B, two populations of $^{35}$S-PEX5L displaying different protease susceptibilities were detected in this experiment, as expected (199, 200, 211). The most abundant at time zero of the export step is the so-called stage 3 PEX5L, a DTM-embedded monoubiquitinated species that leaves the peroxisome very rapidly in the presence of ATP (Figure 14B, compare lanes 0' and 2'; see also (199, 211) and legend to Figure 14B for additional details regarding the properties of peroxisomal PEX5L). The other population is stage 2 PEX5L (the precursor of stage 3 PEX5L), a non-ubiquitinated species that is cleaved at its N terminus by the protease used in these assays yielding a 2-kDa shorter protein. Due to the fact that the buffer used in the export step lacked ubiquitin and components of the ubiquitin-conjugating cascade, the majority of stage 2 PEX5L was not converted into stage 3 PEX5L and therefore remained in the organelles. Densitometric analyses of autoradiographs revealed that about 70% of total peroxisomal $^{35}$S-PEX5L left the organelle in the first 2 min of the export incubation (Figure 14B, lower panel).

Importantly, the export kinetics of $^{35}$S-PEX7 is considerably slower, a difference particularly evident at the 2-min time point of the export assay. Apparently, when PEX5 is exported from the peroxisome it leaves behind a fraction of PEX7, a finding strongly suggesting that export of the two proteins is not coupled. In summary, the data in Figure 13 and 14 suggest that at least a fraction of PEX7 and PEX5 leave the peroxisome separately but through the same site; the finding that no peroxisomal PEX7 is exported whenever PEX5 is arrested at the DTM suggests that DTM-embedded PEX5 behaves as a plug blocking the release of peroxisomal PEX7 into the cytosol (see also Discussion).

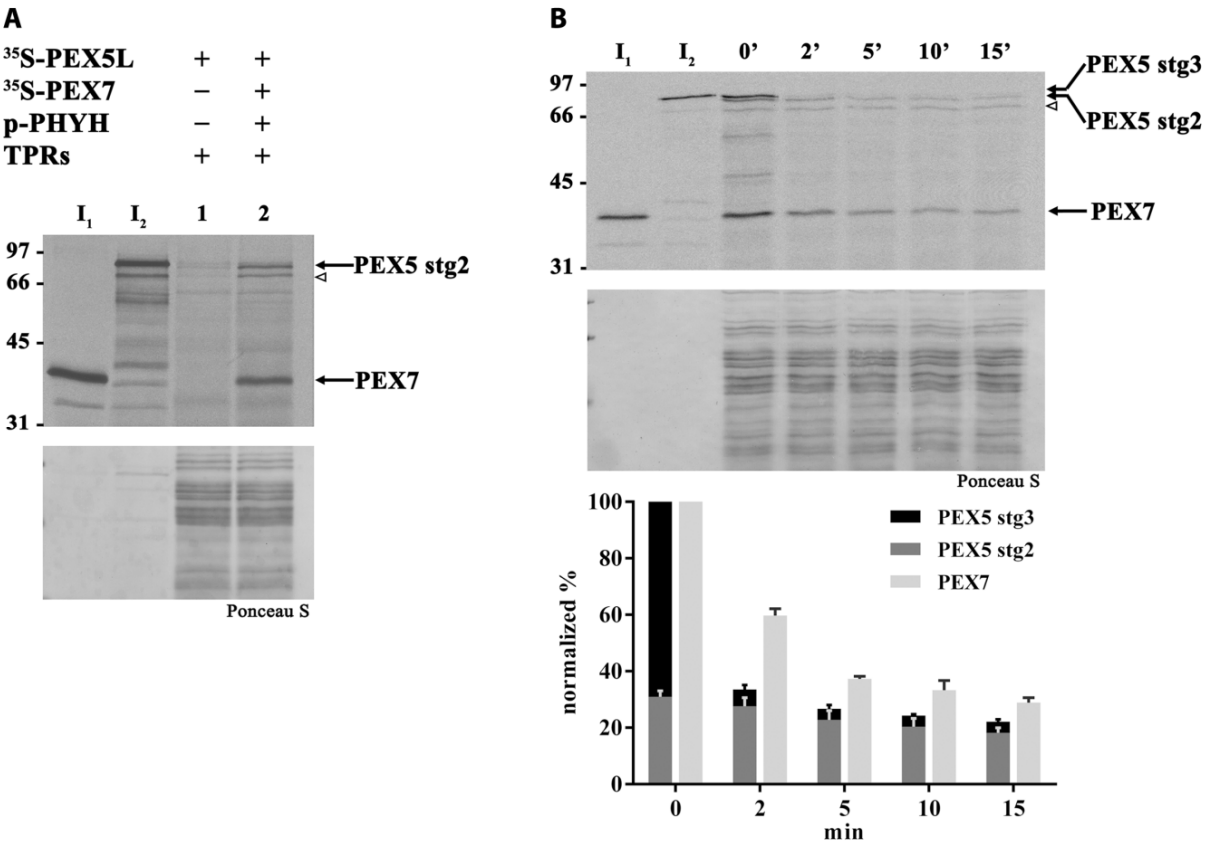


**Figure 14. Peroxisomal PEX5L and PEX7 display different export kinetics.**
**A**, Targeting of PEX5L to the peroxisome in a PTS2-only *in vitro* import system. A reticulocyte lysate containing $^{35}$S-PEX5L was pre-incubated with either a mock-translated lysate (lane 1) or a lysate containing $^{35}$S-PEX7 plus 0.5 µg of p-PHYH (lane 2). Each mixture was then subjected to import assays using PNS supplemented with ATP and 1 µM recombinant TPRs, the PTS1-binding domain of PEX5. After pronase treatment, organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. Lanes $I_1$ and $I_2$, 5% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-PEX5L used in the assays, respectively. **B**, Peroxisomal PEX5L and PEX7 are exported with different kinetics. A mixture of $^{35}$S-PEX7 and $^{35}$S-PEX5L pre-incubated with recombinant p-PHYH was subjected to a 15 min import assay using TPRs-treated PNS in the presence of AMP-PNP. The reaction was diluted with ice-cold import buffer,

and the organelles were isolated by centrifugation, resuspended in import buffer and subjected to an export assay in the presence of ATP, TPRs and NDPEX14. Aliquots were collected at the indicated time points. Pronase-treated organelles were analyzed as in A. Lanes $I_1$ and $I_2$, 2% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-PEX5L used in the assays, respectively. The bar graph shows averages and standard deviations (n=3) of the amounts of peroxisomal $^{35}$S-PEX7, stage 2 $^{35}$S-PEX5L (PEX5 stg2) and stage 3 $^{35}$S-PEX5L (PEX5 stg3) at each time point. Stage 2 PEX5 is converted into stage 3 PEX5 by monoubiquitination at its cysteine 11. The two populations display different susceptibility to proteases: stage 2 PEX5 is cleaved near the N terminus yielding a 2-kDa shorter protein, whereas stage 3 PEX5 is completely resistant because the N-terminal domain is protected by the covalently attached ubiquitin moiety. Note that stage 3 PEX5L runs exactly as unmodified full-length PEX5L upon SDS-PAGE under reducing conditions because the PEX5-ubiquitin thiolester linkage is destroyed by DTT. The open arrow head in A and B indicates an export-incompetent N-terminally truncated PEX5L species produced in the *in vitro* transcription/translation reactions (see also (209)). This species also serves as an internal negative control in the export assay.

## 5. Peroxisomal PEX5 engaged in the PTS2-import pathway remains tightly bound to the organelle membrane.

All the presently available data suggest that PEX5 shuttles between the cytosol and the peroxisomal DTM where it acquires a transmembrane topology, without ever entering completely into the organelle matrix (200, 202, 203, 211, 258). However, it is important to note all those data were obtained with experimental systems in which PEX5 is mostly involved in the PTS1-mediated protein import pathway. Considering a previously proposed hypothesis that PEX20, the yeast functional counterpart of PEX5, may enter completely into the organelle matrix together with PEX7 (214), it might be possible that mammalian PEX5 functioning in the PTS2-import pathway also follows a similar route. To address this possibility we used the PTS2-dependent import assay described above and tried to determine whether $^{35}$S-PEX5L co-fractionates with either membrane or matrix peroxisomal proteins. Briefly, protease-treated organelles from import assays made in the presence of ATP or AMP-PNP were disrupted by sonication and subjected to ultracentrifugation to separate membrane from soluble proteins. The efficiency of this procedure was assessed by monitoring the behavior of catalase, a peroxisomal matrix protein (2, 259) and PEX13, an intrinsic peroxisomal membrane protein and a component of the DTM (260). As shown in Figure 15A, $^{35}$S-PEX5L quantitatively co-fractionated with the membrane marker

PEX13, thus strongly suggesting that peroxisomal PEX5 engaged in the PTS2-protein import pathway remains tightly bound to the peroxisomal membrane. A different result was obtained for PEX7. Indeed, although a major fraction of $^{35}$S-PEX7 was found in the membrane pellet some protein was also detected in the soluble fraction. A similar distribution was observed for endogenous rat liver PEX7 present in highly pure peroxisome preparations (Figure 15B).

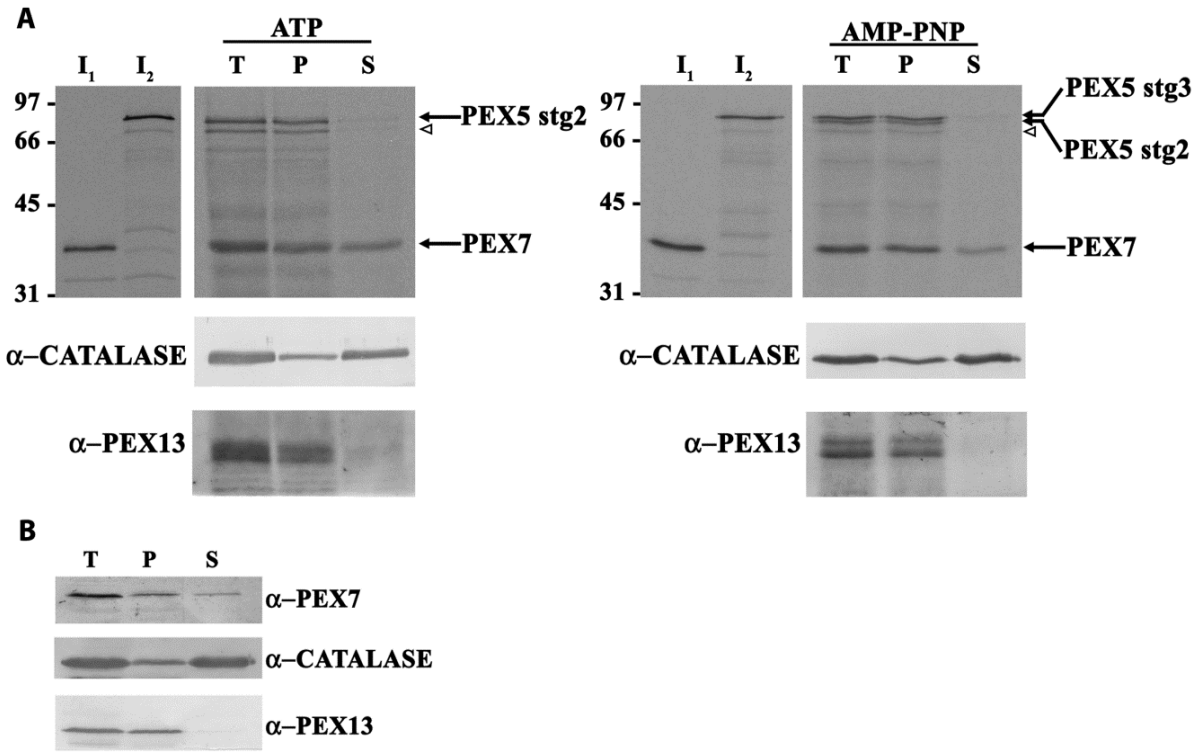


**Figure 15. Peroxisomal PEX5L engaging in PTS2 import remains tightly bound to the peroxisomal membrane, whereas a fraction of PEX7 behaves as a matrix protein.**
**A**, A mixture of $^{35}$S-PEX7 and $^{35}$S-PEX5L pre-incubated with p-PHYH was subjected to an import assay using TPR-treated PNS in the presence of ATP (left panel) or AMP-PNP (right panel), as indicated. After pronase treatment, organelles were disrupted by sonication. Half of the suspension was left on ice (lanes T) while the other half was subjected to ultracentrifugation to obtain membrane (P) and soluble (S) fractions. Samples were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. Lanes $I_1$ and $I_2$, 2% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-PEX5L used in the assays, respectively After autoradiography to detect $^{35}$S-PEX7 and $^{35}$S-PEX5L, the membrane was probed with antibodies against Catalase (α-CATALASE) and PEX13 (α-PEX13). PEX5 stg2 and PEX5 stg3, stage 2 and stage 3 $^{35}$S-PEX5L, respectively. Note that PEX13 is converted into 28- to 30-kDa fragments after protease treatment (201) **B**, An identical sonication experiment was done using rat liver purified peroxisomes. The nitrocellulose membrane was also probed with antibodies against PEX7 (α-PEX7).

The detection of a soluble population of PEX7 in these experiments could very well support the idea that PEX7 is completely released into the matrix of the organelle during the PTS2 import pathway, as previously suggested for the yeast peroxin (186). However, it is also possible that the sonication procedure used here leads to the extraction of proteins that are weakly associated with membranes and so, additional efforts were made to clarify if PEX7 is completely translocated into the organelle matrix or if, like PEX5, is retained at the DTM until the export step takes place.

Considering that each rat liver peroxisome should contain hundreds of DTM complexes (see “Miscellaneous” in Experimental Procedures), we reasoned that it might be possible to co-import two PEX7 species, each of which was pre-incubated with either an export-competent or -incompetent PEX5 protein, and determine how their export capacity is affected by the pre-incubation step. If the two proteins (PEX5 and PEX7) remain associated during their passage through the peroxisome, then the export capacity of a given PEX7 should be determined by the PEX5 protein with which it was pre-incubated. If, on the contrary, PEX7 dissociates from DTM-embedded PEX5 and is indeed released to the organelle lumen, it is only reasonable to assume that PEX7 could be exported back to the cytosol through any one of the many DTMs available, and therefore, independently from whichever PEX5 was used during the pre-incubation step.

Such experiments would be feasible provided that 1) *de novo* formation of ΔC1PEX5L-PEX7 complexes downstream of the pre-incubation step, *i.e.* during the import assay, are kept to a minimum and 2) the half-life of the ΔC1PEX5-PEX7 interaction is relatively large so that PEX7 proteins pre-incubated with different recombinant PEX5 proteins do not exchange partners during the import assay. Figure 16 shows two sets of three chemically identical import reactions programmed with two versions of PEX7 that can be easily resolved by SDS-PAGE, PEX7 and His-tagged PEX7 (lanes $I_1$ and $I_2$, respectively). The first set of reactions (lanes 1-3) contained standard amounts of recombinant p-PHYH and ΔC1PEX5L (*i.e.*, 500 ng and 100 ng, respectively) whereas in the second set (lanes 4-6) the amounts of both recombinant proteins were decreased 10-fold. The only difference between reactions in each set regards the way how radiolabeled PEX7 proteins and recombinant ΔC1PEX5L and p-PHYH were handled before starting the import reactions. In one assay the proteins were added to import

reactions individually, whereas in the other two import reactions one of the two $^{35}$S-labeled PEX7 versions was pre-incubated with ΔC1PEX5L and p-PHYH before starting the assay. As shown in Figure 16, the total amounts of imported PEX7 proteins in each of the three reactions containing standard amounts of ΔC1PEX5L and p-PHYH do not vary much (lanes 1-3), suggesting that the formation of trimeric complexes during the import assay is a relatively fast event under these conditions. Nevertheless, it is already apparent that pre-incubating a given PEX7 protein with the recombinant proteins provides some kinetic advantage to that PEX7 species in the subsequent import assay (compare lane 1 with lanes 2 and 3), suggesting that the ΔC1PEX5L-PEX7 interaction is relatively stable. The kinetic advantage provided by the pre-incubation step becomes particularly evident in assays containing 10-fold less recombinant proteins (compare lane 4 with 5 and 6). Apparently, assembly of trimeric complexes during the import reaction becomes now a rate-limiting event.

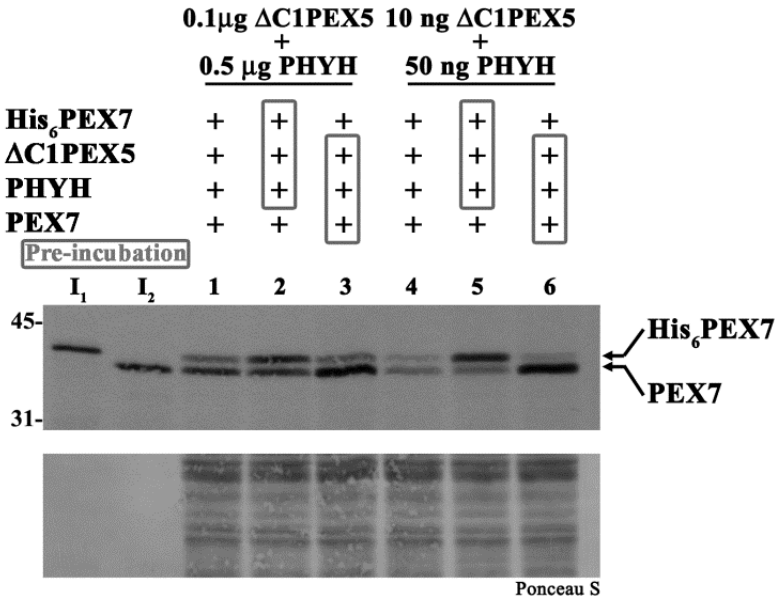


**Figure 16. Co-import assays of $^{35}$S-His$_6$PEX7 and $^{35}$S-PEX7. When PEX5L and p-PHYH are limiting, the pre-incubation step determines which PEX7 is efficiently imported.**
Two sets of three chemically identical co-import reactions were performed with the indicated amounts of ΔC1PEX5L and p-PHYH (lanes 1, 2, 3 and lanes 4, 5, 6, respectively). The recombinant proteins were either added individually (lanes 1 and 4) or they were pre-incubated with one of the two reticulocyte lysates containing PEX7 (lanes 2, 5 and 3, 6 for $^{35}$S-His$_6$PEX7 and $^{35}$S-PEX7 respectively) before being added to the co-import reaction also containing the other $^{35}$S-labeled PEX7 species. Import was allowed to occur for 5 min at 23 ºC. Pronase-treated organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. Lanes $I_1$ and $I_2$, 2.5% of the reticulocyte lysates containing $^{35}$S-labeled His$_6$PEX7 and PEX7 used in each reaction.

It is important to note that under these conditions the concentration of recombinant ΔC1PEX5L is comparable to the one of endogenous rat liver PEX5 (approximately 30 ng per reaction; (175)). This implies that any $^{35}$S-labeled PEX7 that did not associate with ΔC1PEX5L during the pre-incubation step or, that having done so, dissociates from the recombinant protein during the import assay, may form a complex with endogenous PEX5 and, subsequently, be imported into and exported from peroxisomes.

Next, we performed import/export assays with the two $^{35}$S-labeled PEX7 proteins, each of which was individually pre-incubated with recombinant p-PHYH and either ΔC1PEX5L or export-incompetent ΔC1PEX5L(C11A). As shown in Figure 17, when both PEX7 proteins were pre-incubated with ΔC1PEX5L(C11A), approximately 30% of each radiolabeled protein was exported after a 15-min incubation in the presence of ATP (upper panel, lanes 5 and 6; see also lower panel for a quantification of the data). As explained above, it is possible that endogenous rat PEX5 is more of an interfering factor in these assays than on those depicted in Figure 13 where the export efficiency in the presence of ΔC1PEX5L(C11A) rated only at 15% (see Figure 13D). Importantly, when ΔC1PEX5L was used in the pre-incubation step, the amounts of exported $^{35}$S-labeled PEX7 proteins, be it His-tagged PEX7 (Figure 17, lanes 1 and 2) or untagged PEX7 (lanes 3 and 4), were now almost two-fold larger. Thus, the export competence of peroxisomal PEX7 is largely determined by the PEX5 protein with which it associated prior to import. This finding strongly suggests that the PEX5-PEX7 interaction is preserved during the passage of this protein complex through the peroxisome and is only disrupted when PEX5 is exported back into the cytosol. These results strongly support a model in which at least a major fraction of PEX7 enters and exits the peroxisome through the same DTM, suggesting therefore, that PEX7 is never released into the peroxisomal matrix (see Discussion).

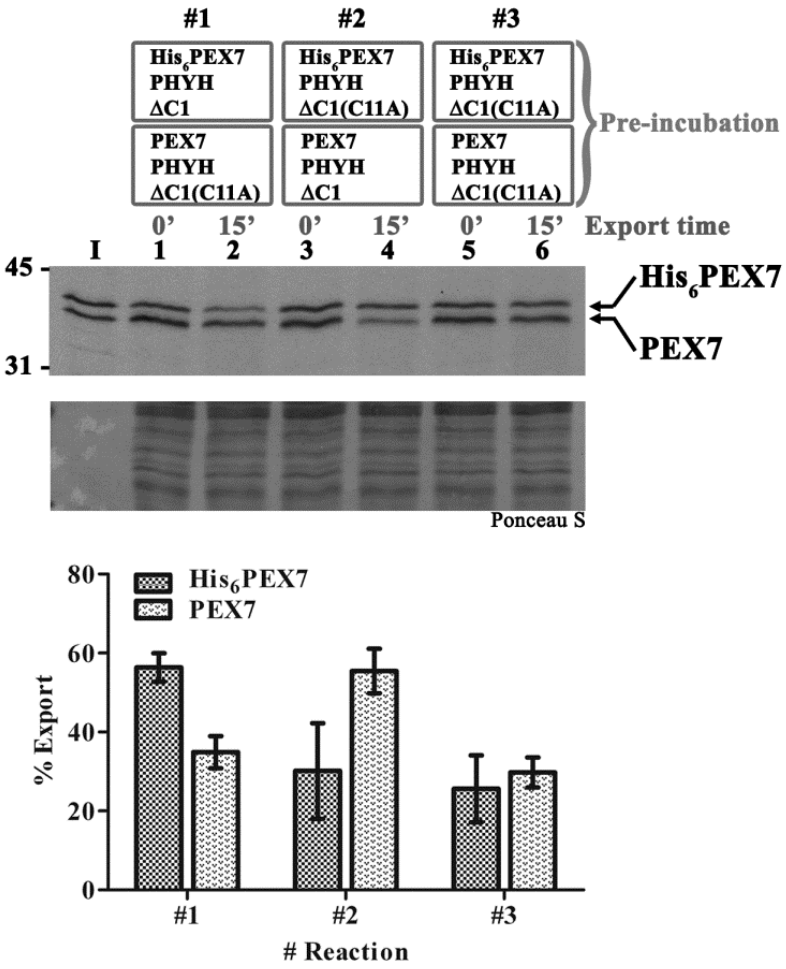


**Figure 17. Pre-incubation of PEX7 with export-competent or -incompetent PEX5 pre-determines its export capability.**

Each reticulocyte lysate ($^{35}$S-His$_6$PEX7 or $^{35}$S-PEX7) was pre-incubated individually with 25 ng of p-PHYH and 5 ng of ΔC1PEX5L or ΔC1PEX5L(C11A) for 20 min at 23 ºC. These mixtures were added to a rat liver PNS in import buffer containing ATP in three different import configurations: (#1) Co-import of $^{35}$S-His$_6$PEX7 and $^{35}$S-PEX7 pre-incubated with ΔC1PEX5L and ΔC1PEX5(C11A), respectively. (#2) Co-import of $^{35}$S-His$_6$PEX7 and $^{35}$S-PEX7 pre-incubated with ΔC1PEX5(C11A) and ΔC1PEX5L, respectively. (#3) Co-import of $^{35}$S-His$_6$PEX7 and $^{35}$S-PEX7, both pre-incubated with ΔC1PEX5(C11A). Import was allowed to occur for 5 min at 23 ºC. At this point, import was inhibited by the addition of NDPEX14 (30 μM) and the reaction further incubated at 37 ºC. Aliquots were taken at the indicated time points. Pronase-treated organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. Autoradiograph and the corresponding Ponceau S-stained membrane are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa. Lane I, 2.5% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-His$_6$PEX7 were mixed and loaded together in the same lane. The bar graph shows averages and standard deviations (n=4) of the percentage of PEX7 export for the different co-import reactions.

## 6. Export of PEX7 from the DTM does not require processing of the PTS2 cargo it transport.

Previous work from Alencastre et al. revealed that PTS2 proteins are released to the peroxisomal matrix before PEX5 ubiquitination (198). The same conclusion was recently extended to PTS1 proteins (196). Interestingly, it has also been proposed that binding of PEX14 to cargo-loaded PEX5 triggers the release of PTS1 proteins into the lumen of the organelle (175). *A priori*, a similar mechanism could also be valid for PTS2 proteins. However, considering that PTS2 proteins interact strongly with PEX7, and not with PEX5, and that the PTS2-containing peptide is rapidly removed upon arrival of the precursor protein to the peroxisomal matrix, a different mechanism for the PTS2-cargo release is also possible. For instance, and as proposed recently (261), it might be that DTM-bound PEX7 presents the PTS2 protein to Tysnd1, the PTS2-processing peptidase, and that cleavage of the signaling sequence leads to cargo release.

To address this hypothesis, we checked if blocking PTS2 protein maturation would cause PEX7 to be retained at the DTM by impairing its recycling back to the cytosol. We first performed import assays with $^{35}$S-PEX7 and $^{35}$S-p-PHYH to compare the behaviors of wild-type p-PHYH with that of a mutant version where Pro29 and Thr30, the residues corresponding to the -2 and -1 sites of the proteolytic cleavage by Tysnd1 (262), were deleted (p-PHYH(Δ29-30)). As shown in Figure 18, while both radiolabeled p-PHYH and p-PHYH(Δ29-30) were efficiently imported to peroxisomes, only wild-type p-PHYH was processed into its mature form, as predicted.

Interestingly, both versions of radiolabeled p-PHYH showed somewhat similar import kinectics, accumulating in peroxisomes during the 30 min import assay. $^{35}$S-PEX7 in contrast, accumulates much less, which is in agreement with its role as a cycling receptor. Of course, in this experimental configuration, the import of $^{35}$S-PEX7 and $^{35}$S-p-PHYH is uncoupled in the sense that $^{35}$S-PEX7 reaches peroxisomes mostly carrying endogenous PTS2 proteins. Likewise, $^{35}$S-p-PHYH(Δ29-30) may be targeted to peroxisomes by endogenous PEX7. Therefore, the behavior of $^{35}$S-PEX7 in this experiment is not very informative.

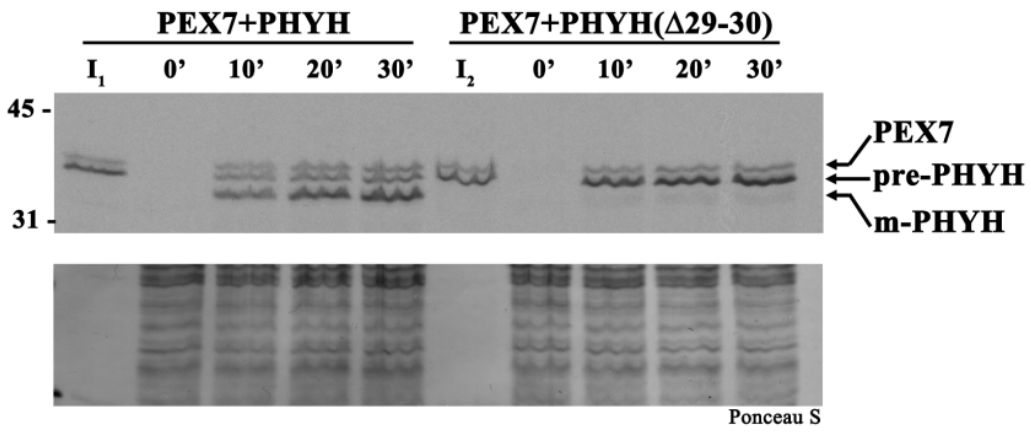


**Figure 18. $^{35}$S-p-PHYH and $^{35}$S-p-PHYH(Δ29-30) are both imported to peroxisomes, but the Δ29-30 amino acid deletion effectively inhibits p-PHYH processing into its mature form (m-PHYH).**

A rat liver PNS was incubated with $^{35}$S-labeled PEX7 and either wild-type $^{35}$S-PHYH or the mutant $^{35}$S-p-PHYH(Δ29-30) in import buffer containing ATP and recombinant ΔC1PEX5L. Import reactions were allowed to occur for 30 min at 37 ºC. Aliquots were taken at the indicated time points. Pronase-treated organelles were analyzed by SDS-PAGE and blotted onto a nitrocellulose membrane. Lanes $I_1$ and $I_2$, 5% of the reticulocyte lysates containing $^{35}$S-PEX7 and $^{35}$S-p-PHYH or $^{35}$S-p-PHYH(Δ29-30) used in each reaction, respectively. Autoradiograph (upper panel) and corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa.

To really assess the effect that inhibiting the proteolytic cleavage of the PTS2 peptide might have on PEX7 import/export dynamics we performed *in vitro* export assays in the presence of recombinant p-PHYH(Δ29-30), similar to those shown in Figure 13. The amount of recombinant PTS2 protein used in these assays (140 nM; see Experimental Procedures) ensures that the amount of $^{35}$S-PEX7 reaching peroxisomes with endogenous PTS2 proteins is minimal as evidenced by both the stimulatory and inhibitory effects that recombinant p-PHYH has on $^{35}$S-PEX7 and $^{35}$S-thiolase import, respectively (see Figure 5). As shown in Figure 19, when recombinant wild-type p-PHYH is replaced by the mutant p-PHYH(Δ29-30), export of PEX7 back to the cytosol remains mostly unaffected. Thus, cleavage of PTS2-cargo proteins by Tysnd1 is not mandatory for PEX7 export, suggesting that PTS2 protein maturation occurs after release of the precursors into the peroxisomal matrix.

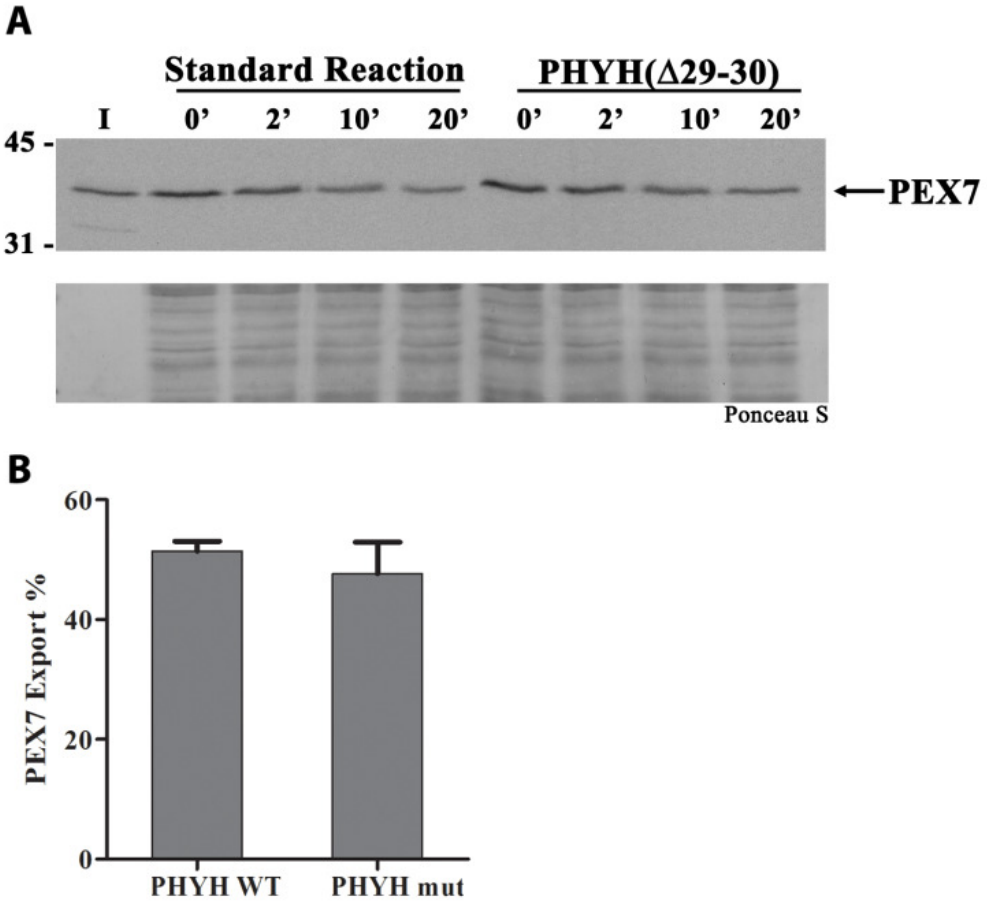


**Figure 19. Export of PEX7 is not affected when the PTS2- cargo protein it transports cannot be processed in the peroxisomal matrix.**

**A**- $^{35}$S-PEX7 was imported for 15 min in the presence of ubiquitin, ATP and the recombinant proteins ΔC1PEX5L and either p-PHYH ("*Standard Reaction*") or p-PHYH(Δ29-30) ("*PHYH(Δ29-30)*"). The reaction mix was then diluted with ice-cold import buffer and the organelles were isolated by centrifugation and subjected to an export assay in the presence of ATP (see Experimental Procedures for details). Aliquots were collected at the indicated time points. Pronase-treated organelles were subjected to SDS-PAGE analysis and blotted onto a nitrocellulose membrane. Lanes I, 5% of the reticulocyte lysate containing $^{35}$S-PEX7 used in each reaction. Autoradiograph (upper panel) and the corresponding Ponceau S-stained membrane (lower panel) are shown. Numbers to the left indicate the molecular masses of the protein standards in kDa. **B**, The bar graph shows the average percentage of PEX7 export after 20 min under the conditions described in A. Standard deviations (n = 3) are also presented.

# V- DISCUSSION

In this work we show that mammalian PEX7 is targeted to the peroxisome in a PEX5L- and PTS2-dependent manner where it acquires resistance to exogenously added proteases. Importantly, both PEX7 and pre-thiolase, a PTS2 protein, reach this protease-protected location in a cytosolic ATP-independent manner ((198, 245) and this work), implying that the PEX7-PTS2 protein complex enters the peroxisome upstream of the first ATP-dependent step of the PEX5-mediated protein import pathway, *i.e.*, prior to monoubiquitination of DTM-embedded PEX5. Additional data presented in this work corroborate this conclusion. As shown in Figures 7B and 10, a mutant version of PEX5 that cannot be monoubiquitinated at the DTM is as functional as the normal protein in promoting peroxisomal import of both PEX7 and pre-thiolase. Clearly, the PEX5-mediated entry of both PEX7 and its cargo into the peroxisome is not linked to monoubiquitination of PEX5 at the DTM. Interestingly, this conclusion contrasts with the views of the so-called “export-driven import model”, a hypothetical mechanism recently proposed for the yeast PEX18/PEX7 system (213, 263). According to this model, monoubiquitination/export of PEX18, a member of the PEX20 family and a functional counterpart of PEX5 in the PTS2 protein import pathway, is mechanically linked to the translocation of PEX7, and presumably its cargo, across the peroxisomal membrane. Seemingly, the different architectures of the PTS2 protein import machineries in these organisms translate into at least some significant mechanistic differences.

One of the aims of this work was to characterize the intraperoxisomal pathway followed by mammalian PEX7 during the PTS2 protein transport cycle. Up till now, there was only one study addressing this problem in a systematic manner. This is a work by Lazarow and colleagues describing the properties of a yeast PEX7-green-fluorescent-protein (GFP) fusion protein, a protein that although unable to complement the phenotype of a Δ*PEX7* strain, accumulates massively in the peroxisomal matrix (186). As shown by those authors, cleavage of the fusion protein at the PEX7-GFP junction yielded a PEX7 protein that could now exit the organelle and rescue the phenotype of the Δ*PEX7* strain. Apparently, there is a way out of the peroxisome for a PEX7 protein that was artificially accumulated in the matrix of the organelle. Based on those findings the authors proposed that PEX7 follows an “extended cycling mechanism”, *i.e.*, that PEX7 enters completely

into the peroxisome matrix during the PTS2 protein transport cycle (186). The results described here for preL4R-PEX7 suggest that at least the N terminus of mammalian PEX7 enters sufficiently deep into the peroxisome matrix milieu to become accessible to the peroxisomal protease that cleaves the engineered pre-sequence. Furthermore, fractionation of organelles by sonication did reveal the existence of a PEX7 pool displaying the properties expected for a peroxisomal matrix protein. Although these two observations are compatible with the “extended cycling” pathway (see Figure 20, pathway A), they do not allow us to formally exclude a model in which PEX7, like PEX5, is retained at the DTM during its passage through peroxisomes (Figure 20, pathway B). Indeed, sub-fractionation of organelles by sonication and centrifugation (the only experiment that would favor the “extended cycling mechanism” over the “retention” model) does have a major caveat: it can lead to the extraction of proteins that are weakly associated with membranes. In an attempt to clarify this important issue, we used our in vitro import/export system to determine whether the export competence of PEX7 is dictated by the export capacity of the PEX5 molecule that transported it to the peroxisome. If PEX7 is indeed released into the peroxisomal matrix, then it should be able to leave the organelle through any one of the hundreds of DTMs present in each peroxisome, regardless of the properties of the PEX5 molecule with which it associated in the cytosol. If on the contrary, PEX7 is retained at the DTM, its export efficiency will be defined by the PEX5 molecule that mediated its import. To assemble [PEX5-PEX7-PTS2] complexes of defined composition we pre-incubated lysates containing the different $^{35}$S-labeled PEX7 with recombinant p-PHYH and either the export competent or incompetent versions of recombinant ΔC1PEX5L. These complexes were shown to be stable and efficiently imported to peroxisomes. The import/export experiments depicted in Figure 17 show that the PEX5 species used in the pre-incubation step (ΔC1PEX5L or ΔC1PEX5L(C11A)) greatly determined the export efficiency of PEX7. In other words, when PEX7 is transported to the peroxisome by an export-competent PEX5 species, then PEX7 becomes also export competent. In contrast, when PEX7 is transported to the organelle by an export-incompetent PEX5 molecule, then its subsequent export is also compromised. Although further data should be collected, these results strongly support a scenario where PEX7, traveling through the peroxisome, is

never released into the matrix and, like PEX5, is retained at the DTM until the extraction step (see pathway B in Figure 20).

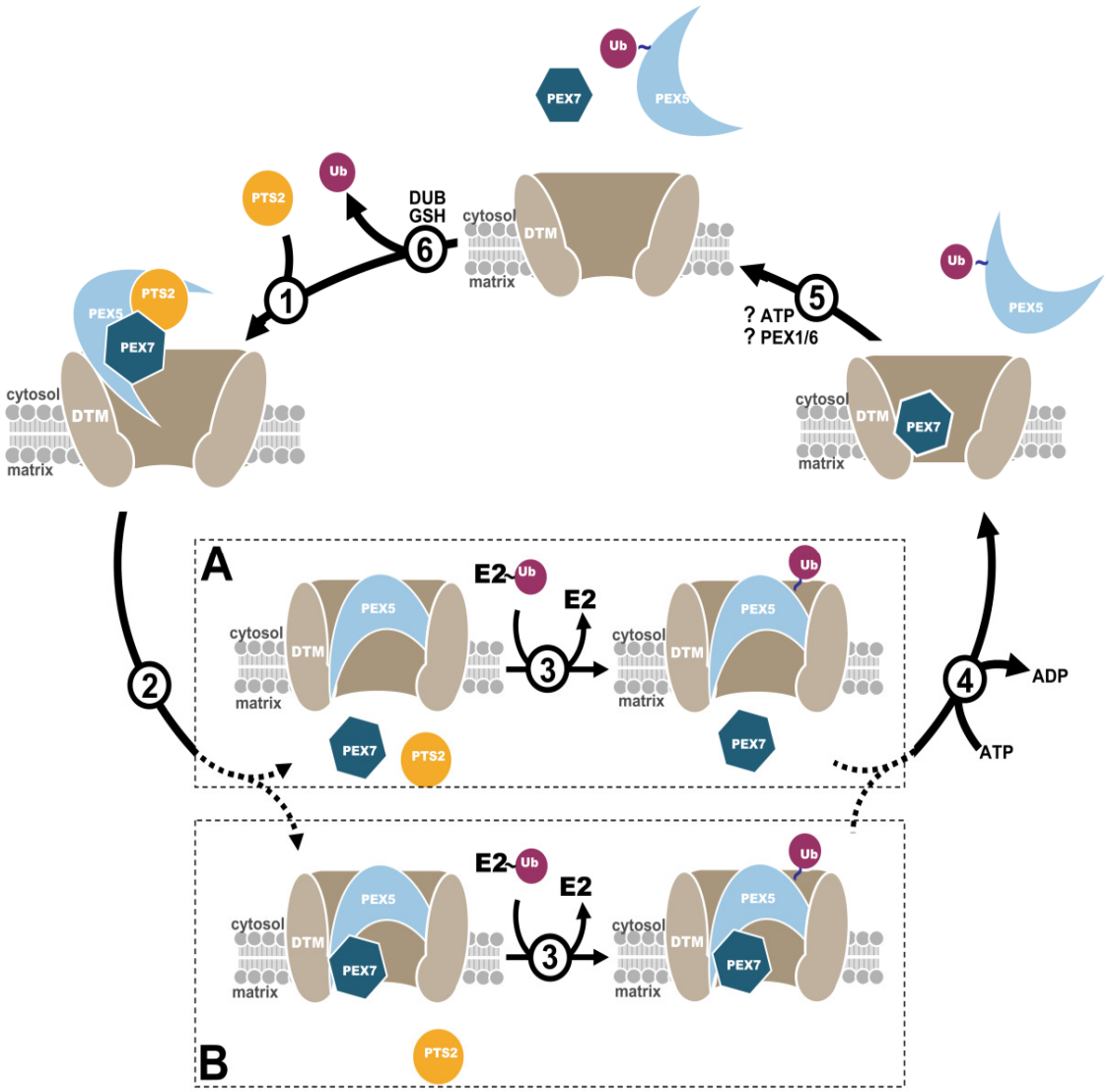


**Figure 20. Working model for the PEX5-PEX7-mediated import pathway.**

After its assembly in the cytosol, the trimeric PEX5-PEX7-PTS2 protein complex docks at the docking/translocation machinery (DTM) [arrow 1]. This receptor-cargo complex then becomes inserted into the DTM [arrow 2]. This step culminates with the PTS2 cargo protein being delivered to the organelle matrix (where the PTS2 is cleaved) and PEX5 displaying a transmembrane topology (*i.e.*, stage 2 PEX5). At this stage, PEX7 is completely protected from exogenous proteases exposing at least its N terminus to the peroxisome matrix. PEX7 could be completely released from the DTM into the matrix milieu (pathway A) or may be retained at the DTM until the export step (pathway B). Following insertion into the DTM, PEX5 is monoubiquitinated at the conserved cysteine 11 residue [arrow 3], yielding stage 3 PEX5. Monoubiquitination of PEX5 allows its ATP-dependent extraction from the DTM [arrow 4], and the subsequent export of PEX7 [arrow 5]. After deubiquitination of PEX5 [arrow 6], the protein transport cycle restarts.

Many important aspects of the PTS2-mediated protein import pathway remain unclear. One directly related to this work regards the molecular details of PEX7 export. Our data suggest that PEX7 leaves the peroxisomal compartment through the DTM site occupied by PEX5 and that peroxisomal PEX5 and PEX7 probably exit the organelle separately. Our data clearly shows that PEX7 export from the peroxisome requires PEX5-free DTMs and therefore the action of the mechanoenzymes PEX1 and PEX6. Thus, these ATP-dependent enzymes surely influence PEX7 export, but whether this functional connection is merely indirect (*i.e.*, via PEX5 export) or direct remains to be determined. Regardless, it is likely that the ATP-dependent extraction of PEX5 from the DTM also disrupts the interaction between PEX5 and PEX7, thus preparing PEX7 for a new PTS2-recognition event.

In this work we developed a strategy allowing us to analyze the trafficking of a PEX5L population engaged exclusively in the import of PTS2 proteins. The data collected suggest that PTS1- and PTS2-mediated import of mammalian peroxisomal matrix proteins are quite similar. First, it is now clear that the targeting of PEX5L to the peroxisomal membrane is in both cases cargo-dependent, be it a PTS1 cargo (202) or a [PTS2-PEX7] complex. Secondly, in both pathways, insertion of PEX5L into the DTM results in the exact same transmembrane topology for PEX5, with the protein exposing a 2-kDa fragment of its polypeptide chain to the proteases used in these *in vitro* import assays (see PEX5 stg2 in Figures 14 and 15). Finally, the receptors are recycled back into the cytosol in a process that depends on PEX5 monoubiquitination and extraction by the ATP-dependent REM. The fast export kinetics we observe for monoubiquitinated PEX5L in our PTS2-only assays (see PEX5 stg3 in Figure 14B) is also compatible with the kinetics previously observed in *in vitro* assays where PEX5L was working predominantly on the import of PTS1 proteins (199). An aspect where PTS1 and PTS2 import might diverge regards the cargo release step. We have tried to implicate the proteolytic cleavage of the PTS2 pre-sequence as a possible trigger for PTS2-cargo release into the peroxisomal lumen. An association between these two events seemed likely. First, the relevance of PTS2 protein maturation is poorly understood as both the precursor and processed form of these enzymes appear to

be equally active (264, 265). Additionally, removing the targeting signal from the PTS2 protein would be an effective way to abrogate its interaction with PEX7, which in turn would result in its release into the peroxisomal matrix. In our attempts to clarify this issue, we used a mutated version of p-PHYH with a deleted cleavage site (p-PHYH(Δ29-30)). Although this mutation effectively inhibited the proteolytic cleavage of the PTS2 pre-sequence, this protein showed a normal accumulation in peroxisomes and the export of PEX7 did not seem to be affected. Although we did not find evidence to suggest that PTS2 protein maturation triggers cargo release, or affects PEX7 recycling, we should note that the absence of Tysnd1 in mice does cause a phenotype that resembles that of a mild Zellweger spectrum disorder (261). Apparently, Tysnd1 contributes to a normal peroxisome function but, according to our results, its role is probably not linked to the biogenesis of the organelle.

Interestingly, very recent pull-down assays with *Arabidopsis* recombinant proteins showed that thiolase no longer interacts with PEX7 and PEX5 when PEX14 is added to the assays (266). This observation is comparable to the data previously described for human proteins showing that PEX14 disrupts PEX5-catalase interaction (175). These results give strength to the notion that the interaction between PEX5 and DTM components (*e.g.* PEX14) is sufficient to trigger release of the cargoes into the peroxisomal matrix (156, 175, 196, 198, 267).

Although this work has clarified many aspects of the PTS2-mediated import pathway, it is clear that further work is necessary to completely understand many of the details of this machinery. As stated earlier, the direct action of the AAA ATPases PEX1 and PEX6 on PEX7 export cannot be formally excluded at the moment. Another question that we have yet to address regards the protein transport capacity of PEX5L. Can a single molecule of PEX5L simultaneously transport a PTS1 and a PTS2 protein to the peroxisome, or are these mutually exclusive events? While there have been studies where a tetrameric complex [PTS1-PEX5-PEX7-PTS2] was successfully assembled (266, 268), whether the docking/translocation machinery is capable to accommodate such complex and productively translocate both cargo-proteins remains to be verified. In this work, we developed an improved *in vitro* import system that can be used to further study

the PEX7-mediated import of PTS2 proteins and provide answers to these questions. This *in vitro* system will, in the near future, allow us to easily establish a correlation between PEX7 structure and function by studying the many mutations described in RCDP type 1 patients. For example, import assays using postnuclear supernatants from *PEX7* knockout mice, together with other biochemical approaches (*e.g.,* native-PAGE analyses, size-exclusion chromatography), will allow us to distinguish between defects in cargo-binding, PEX5 binding, DTM association and defects in the recycling step of the receptor. Such analysis could also lead to the identification of mutations that only affect PEX7 folding and stability. RCDP type I patients with this type of mutations could perhaps benefit from some form of chaperone therapy, an approach that has shown some promise when treating lysosomal storage diseases caused by the so-called chaperone-responsive mutations (reviewed in (269, 270)).

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