Charakterisierung der späten Schritte des peroxisomalen Matrixprotein Imports in *Saccharomyces cerevisiae*

DISSERTATION

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ZUSAMMENFASSUNG

Im Rahmen der vorliegenden Arbeit ist eine funktionelle Analyse der sogenannten späten Schritte des peroxisomalen Matrixproteinimports in der Bäckerhefe *Saccharomyces cerevisiae* durchgeführt worden. Den Hintergrund für die dokumentierten Experimente bildete die Beobachtung, dass der PTS1-Rezeptor Pex5p während des Imports von peroxisomalen Matrixproteinen zwischen dem Zytosol und dem peroxisomalen Kompartiment pendelt. Während Daten zur Anbindung von Pex5p an die Membran so wie auch für dessen Weitergabe an verschiedene Membrankomplexe existieren, waren Faktoren und Mechanismus der Export-Reaktion vollkommen unverstanden. Auf diesem letzten Schritt lag der Schwerpunkt dieser Arbeit.

Der PTS1-Rezeptor Pex5p wird auf zwei unterschiedliche Arten ubiquitinyliert. So konnte in dieser Arbeit erstmalig nachgewiesen werden, dass Pex5p mit K48-verzweigten Polyubiquitinketten modifiziert wird. Polyubiquitinylierung tritt in Mutanten des AAA-ATPase-Komplexes sowie des Pex4p/Pex22p-Komplexes auf und erfolgt an der peroxisomalen Membran in Abhängigkeit von den RING-Finger Peroxinen. Die mögliche Funktion der RING-Finger Pex2p, Pex10p und Pex12p als Ubiquitin-Protein-Isopeptid Ligasen wird diskutiert. Anhand einer systematischen Analysereihe von Deletionsmutanten konnte das Ubiquitin-konjugierende Enzym Ubc4p als zentrales E2-Enzym der Polyubiquitinylierung von Pex5p identifiziert werden, dessen Funktion teilweise von Ubc5p oder Ubc1p übernommen werden kann. Als Resultat der gerichteten Mutagenese aller Lysin-Reste in der amino-terminalen Hälfte von Pex5p konnten die ersten beiden Lysine, K18 und K24, als Ankerreste der Polyubiquitinketten identifiziert werden. Das polyubiquitinylierte Pex5p wird im 26S Proteasom degradiert, was diese Art der Modifikation des Rezeptors als Form einer Peroxisomen-spezifischen Qualitätskontrolle charakterisiert.

Die zweite Ubiquitinylierungsform von Pex5p ist die Monoubiquitinylierung unter Wildtyp Bedingungen. Diese Form akkumuliert nicht, sondern stellt eine transiente Modifikation dar, die nur bei chemischer Inhibierung der Deubiquitinylierenden Enzyme detektierbar ist. Auch diese Form der Modifikation erfolgt an der peroxisomalen Membran in Abhängigkeit von den RING-Finger Peroxinen. Die Stelle für das Anheften des Ubiquitins auf Pex5p ist noch nicht identifiziert, jedoch ist es klar, dass es sich hierbei um andere Reste als bei der Polyubiquitinylierung handelt. Monoubiquitinylierung von Pex5p ist unabhängig von den nicht-peroxisomalen Ubcs und benötigt die Aktivität des peroxisomalen E2-Enzyms Pex4p.

Im Rahmen dieser Arbeit ist erstmals ein zellfreies *in vitro* Export System etabliert worden, um die mechanistische Bedeutung und Funktion der AAA-Peroxine, von Pex4p sowie der Ubiquitinylierung von Pex5p aufzuklären. Hierbei konnte klar demonstriert werden, dass die peroxisomale Lokalisation wie auch die ATPase Aktivität von Pex1p und Pex6p essentiell für die Freisetzung des PTS1-Rezeptors sind. Die von Pex4p katalysierte Monoubiquitinylierung von Pex5p stellt allem Anschein nach das primäre Signal für den Export von Pex5p durch die AAA-Peroxine dar. Der unter Pex4p-defizienten Bedingungen beobachtbare Rest-Export von Pex5p ist Polyubiquitin-abhängig, da Pex5p(K18R;K24R) nicht mehr abgelöst wird. Somit ist die Ubiquitinylierung neben der Aktivität der AAA-Peroxine essentiell für den Export Schritt des PTS1-Rezeptors. Der Energieverbrauch dieser Reaktionsfolge kann somit in zwei Schritte differenziert werden: Pex4p-abhängige Rezeptor-Ubiquitinylierung und AAA-abhängige Dislokation.

1. EINLEITUNG

1.1 Struktur und Funktion von Peroxisomen

Peroxisomen sind Organellen der als *Microbodies* zusammengefassten Klasse an Zellkompartimenten. Ihr namensgebendes Charakteristikum besteht darin, dass sie mindestens eine Wasserstoffperoxid-produzierende Oxidase, sowie eine detoxifizierende Katalase, welche das peroxisomale Leitenzym darstellt, aufweisen (De Duve & Baudhuin, 1966). Die weiteren zu den *Microbodies* zählenden Organellen werden aufgrund ähnlicher enzymatischer und strukturproteinogener Ausstattung häufig als spezialisierte Formen der Peroxisomen angesehen (Thieringer & Kunau, 1991). Zu ihnen zählen zum einen die Glyoxysomen der Pflanzenkeimlinge und niederer Pilze, welche Enzyme des Glyoxylat-Zyklus zur Umwandlung von Lipiden in Kohlenhydrate beherbergen (Breidenbach & Beevers, 1967; Kionka & Kunau, 1985). Des weiteren besitzen filamentöse Pilze oftmals zusätzlich zu anderen *Micobodies* die sog. Woronin-Körper, die zur Verschließung septaler Poren nach Beschädigung der Hyphen dienen, um das Austreten des Zytoplasmas zu verhindern (Woronin, 1864; Jedd & Chua, 2000). Die Glykosomen der Trypanosomen beherbergen Enzyme der Glykolyse (Opperdoes & Borst, 1977). *Microbodies* sind metabolisch dadurch gemeinsam definiert, dass sie Enzyme zum Abbau von Fettsäuren beinhalten (zur Übersicht van den Bosch *et al*., 1992; Eckert & Erdmann, 2003).

Peroxisomen werden von einer einfachen Membran begrenzt und sind von zumeist sphärischer Form mit einem Durchmesser von 0,1 bis 1 µm. Im Gegensatz zu Mitochondrien und Chloroplasten enthalten sie keine eigene DNS. Ihre Anzahl in der Zelle variiert erheblich zwischen wenigen wie z.B. in der Bäckerhefe und einigen Hunderten in Hepatocyten von Säugern. Ebenso vielfältig ist die morphologische und biochemische Variation von Peroxisomen zwischen den verschiedenen Spezies. Sie verfügen je nach Gewebe und Funktion über eine unterschiedliche Ausstattung an Membran- und Matrixproteinen und werden demzufolge auch als „multifunktionelle Organellen“ bezeichnet (Opperdoes, 1988).

Die in der peroxisomalen Matrix enthaltenen Enzyme sind in verschiedene Stoffwechselwege eingebunden. Dazu zählt vor allem die β-Oxidation von Fettsäuren, welche bei den niederen Pilzen (Kunau *et al.*, 1988) und den Pflanzen (Gerhardt, 1992; Kindl, 1993) ausschließlich in den Peroxisomen lokalisiert vorliegt, wohingegen bei Säugetieren nur der Abbau langkettiger Fettsäuren in den Peroxisomen stattfindet (Lazarow & De Duve, 1976). Die α-Oxidation von Fettsäuren ist in Säugern und Pflanzen peroxisomal lokalisiert (zur Übersicht Graham & Eastmond, 2002; Jansen & Wanders, 2006). Die Peroxisomen der Pflanzen können in einer

der ß-Oxidation parallel verlaufenden Reaktionsfolge die Phytohormone Indol-3-essigsäure, Jasmonsäure und Salicylsäure aus entsprechenden Vorstufen bilden (zur Übersicht Nyathi & Baker, 2006; Pollmann *et al.*, 2006) und sind außerdem an der Photorespiration beteiligt (zur Übersicht Johnson & Olsen, 2001). In einigen filamentösen Pilzen finden hier die letzten Reaktionsschritte der Penizillin-Biosynthese statt. (Müller *et al.*, 1991). Als zusätzliche Funktionen im Metabolismus von zoologischen Organismen sind die Plasmalogensynthese, Teile der Gallensäure- und Cholesterinbiosynthese, der Prostaglandinstoffwechsel und der Polyamin- und möglicherweise Purinmetabolismus zu nennen (zur Übersicht van den Bosch *et al.*, 1992; Parsons *et al.*, 2001; Hiltunen *et al.*, 2003; Wanders, 2004).

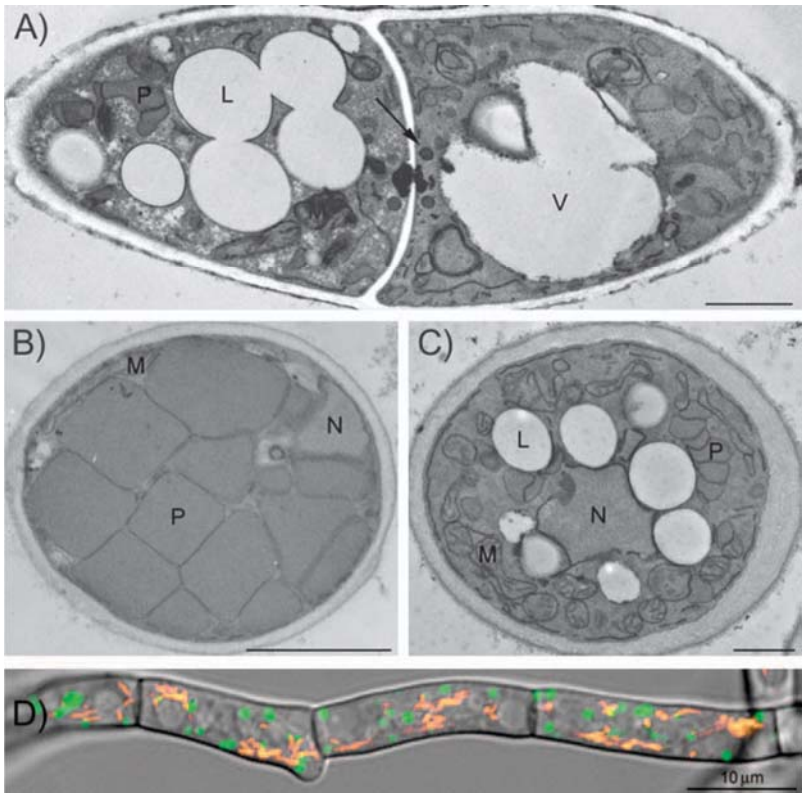

Abb. 1.1 Induktion von Peroxisomen in Hefen und filamentösen Pilzen. Peroxisomen können in sehr variabler Form und Verteilung in der Zelle vorkommen. A) EM-Bild einer *Aspergillus tamarii* Zelle, die auf Oleat gewachsen ist und neben Peroxisomen zusätzlich Woronin-Körper in der Nähe des Septums aufweist (Pfeil). B) *Hansenula polymorpha* nach der Induktion der Proliferation von Peroxisomen durch Methanol-limitiertes Wachstum. Mehr als 80 % der Zelle sind mit Kubus-förmigen Peroxisomen angefüllt.
C) *Saccharomyces cerevisiae* zeigt nach Oleat-Induktion charakteristisch aggregierte Peroxisomen.
D) Fluoreszenzmikroskopische Aufnahme von *Penicillium chrysogenum* Hyphen. Die Peroxisomen sind durch das Markerprotein GFP-PTS1 grün markiert. Die Mitochondrien erscheinen mittels *Mitotracker* orange. Der Balken entspricht 1µm bei den EM Bildern. L=Lipid Tropfen, M=Mitochondrium; P=Peroxisom, V=Vakuole (aus Kiel *et al.*, 2006)

1.2 Peroxisomale Erkrankungen

Peroxisomen spielen aufgrund der Vielzahl an biochemischen Reaktionswegen, die in ihnen lokalisiert sind, eine bedeutende Rolle für den Metabolismus des Menschen. Entsprechend führen Defekte der peroxisomalen Funktion zu schweren Krankheitsbildern. Die Patienten sind u. a. durch schwere neurologische Defekte sowie Nieren- und Leberinsuffizienz gekennzeichnet, die meistens schon kurz nach der Geburt zum Tode der Betroffenen führen. Die peroxisomalen Erkrankungen können auf Grundlage der klinischen Befunde in zwei Gruppen unterteilt werden. Zur Gruppe I zählen Krankheitsbilder, die durch einen generellen peroxisomalen Funktionsverlust hervorgerufen werden und die entsprechend als peroxisomale Biogenese-Erkrankungen (*peroxisome biogenesis disorders*, PBDs) zusammengefasst werden (zur Übersicht Wanders & Waterham, 2005). Erkrankungen der Gruppe II lassen sich auf singuläre peroxisomale Enzymdefekte zurückführen (zur Übersicht Wanders & Waterham, 2006).

Die Erkrankungsformen der Gruppe I werden durch Defekte in verschiedenen peroxisomalen Biogenesefaktoren hervorgerufen und heute zusammenfassend auch als Zellweger-Spektrum bezeichnet (zur Übersicht Wanders & Waterham, 2005). Patientenzellen der Gruppe I können durch die Abwesenheit von morphologisch detektierbaren Peroxisomen gekennzeichnet sein, wobei in den Zellen oftmals noch leere peroxisomale Strukturen (sog. *ghosts*) nachgewiesen werden können (Santos *et al.*, 1988). Das autosomal-rezessiv vererbte Zellweger Syndrom, die zuerst entdeckte menschliche peroxisomale Erbkrankheit, gehört in diese Kategorie (Bowen *et al.*, 1964; Goldfischer, 1973). Des weiteren sind die neonatale Adrenoleukodystrophie (NALD) (Kelley *et al.*, 1986) und die infantile Refsum Krankheit (IRD) (Scotto *et al.*, 1982) beschrieben worden. Der rhizomelischen *Chondrodysplasia punctata* (RCDP) liegt kein genereller, sondern ein partieller Funktionsverlust der noch detektierbaren Peroxisomen zugrunde, welcher durch den Ausfall des Rezeptors nur eines Proteinimportweges bedingt wird (Bravermann *et al.*, 1997; Motley *et al.*, 1997; Purdue *et al.*, 1997). Die Erkrankungen der Gruppe II zeichnen sich durch Einzelenzymdefekte aus (zur Übersicht Wanders & Waterham, 2006). Hier können Proteine wie die Acyl-CoA-Oxidase, oder der peroxisomale ABC-Transporter ALDP betroffen sein. Ein Defekt dieses ABC-Transporters führt zur X-chromosomalen Adrenoleukodystrophie (X-ALD), der häufigsten peroxisomalen Krankheit (Braun *et al.*, 1994). In Bezug auf die Beschreibung der peroxisomalen Erkrankungen ist zu berücksichtigen, dass die klinische Einteilung nicht der genetischen Klassifizierung entspricht. So kann der Ausfall von zwei verschiedenen Genen der Peroxisomen Biogenese zu ähnlichen klinischen Symptomen führen. Somatische

Zellfusionsanalysen zeigten, dass die weltweit vorhandenen Fibroblasten-Zelllinien in 14 Komplementationsgruppen unterteilbar sind. (Wanders & Waterham, 2005). Um peroxisomale Erkrankungen weiter charakterisieren zu können und wirksame Therapien zu entwickeln, müssen neben klinischen Studien vor allem die genetischen und biochemischen Ursachen genauer erforscht werden. Seit Anfang der neunziger Jahre wird die Biogenese von Peroxisomen vor allem unter Verwendung von eukaryontischen Mikroorganismen als Modellsysteme untersucht.

1.3 Identifizierung der Peroxine

Die Grundlage für die Identifizierung der meisten Proteine, die für die Biogenese und Funktion von Peroxisomen essentiell sind, war die Beobachtung, dass die β-Oxidation von Fettsäuren in Hefen ausschließlich peroxisomal lokalisiert ist (Kunau *et al.*, 1988) und zudem, dass die Proliferation von Peroxisomen auf Ölsäure-haltigen Medien induziert werden kann (Veenhuis *et al.*, 1987). Somit wird die Funktionalität des peroxisomalen Metabolismus essentiell für das Wachstum der Hefe-Zellen unter Bedingungen, wenn Ölsäure als einzige Kohlenstoffquelle zur Verfügung steht. Diese Voraussetzungen ermöglichten die Etablierung einer Methode zur Isolierung von Mutanten mit peroxisomalen Defekten (Erdmann *et al.*, 1989). Isoliert wurden Zellen mit einem *onu*-Phänotyp (*oleat-non-utilizer*), die nicht in der Lage waren mit Ölsäure als einziger Kohlenstoffquelle zu wachsen. Zur Ausbildung dieses Phänotyps kommt es bei Defekten in Enzymen der peroxisomalen β-Oxidation (*fox*-Mutanten (*fatty acid oxidation*) oder bei Defekten in Proteinen, die für die Biogenese der Peroxisomen essentiell sind (*pex*-Mutanten (*peroxisomal assembly*). Die für diesen Phänotyp verantwortlichen FOX- und PEX-Gene konnten über funktionelle Komplementation identifiziert werden (Erdmann & Kunau, 1992; Lazarow, 1993). Die Proteine der für die peroxisomale Biogenese kodierenden PEX-Gene werden als Peroxine bezeichnet (Distel *et al.*, 1996), von denen bisher 32 bekannt sind (siehe Tab. 1.1).

Trotz des Vorhandenseins von Charakteristika und Sequenzmotiven, die z.T. vergleichbar zu Proteinen anderer Systeme sind (siehe Tab.1.1) ist die Funktionsanalyse der meisten Peroxine noch nicht abgeschlossen. Die genaue Rolle der jeweiligen Proteine innerhalb der komplexen Biogenese der Peroxisomen ist bisher nur ansatzweise geklärt.

Tabelle 1.1: Übersicht der bisher identifizierten Peroxine

Berücksichtigt wurden ausschließlich die Arbeiten, welche das Gen bzw. Protein näher charakterisieren, so dass reine *in silico* Ansätze ausgeschlossen wurden.

Peroxin	**Lokalisation**	**Strukturmotiv**	**Funktionsbereich**	**Organismus**
Pex1p	LP MGP	AAA-ATPase	Matrixproteinimport; Rezeptor Recycling	*Hp* [1] *Hs* [2] *Pc* [3] *Pp* [4] *Sc* [5]
Pex2p	IMP	RING-Finger	Matrixproteinimport	*Cg* [6] *Hs* [7] *Ld* [8] *Mm* [9] *Pa* [10] *Pp* [11] *Rn* [12] *Sc* [13] *Yl* [14]
Pex3p	IMP		Membranbiogenese; Verankerung von Pex19p	*Hp* [15] *Hs* [16] *Pp* [17] *Sc* [18]
Pex4p	LP, MGP	UBC-Domäne	Matrixproteinimport ; Ubiquitin-Konjugation	*At* [19] *Hp* [20] *Pp* [21] *Sc* [22]
Pex5p	LP, MGP, IMP, IP	TPR	Matrixproteinimport; PTS1-Rezeptor	*At* [23] *Ca* [24] *Cia* [25] *Hp* [26] *Hs* [27] *Ld* [28] *Mm* [29] *Nt* [30] *Os* [31] *Pa* [32] *Pc* [33] *Pp* [34] *Sc* [35] *Tb* [36] *Yl* [37]
Pex6p	LP MGP	AAA-ATPase	Matrixproteinimport Rezeptor Recycling	*At* [38] *Cla* [39] *Ha* [40] *Hp* [41] *Hs* [42] *Os* [43] *Pc* [44] *Pp* [45] *Rn* [46] *Sc* [47] *Tb* [48] *Yl* [49]
Pex7p	LP, IP	WD40	Matrixproteinimport PTS2-Rezeptor	*At* [50] *Gh* [51] *Hs* [52] *Kl* [53] *Mm* [54] *Nc* [55] *Pp* [56] *Sc* [57]
Pex8p	MPG, IP	Leucin-Zipper	Matrixproteinimport; verbindet Docking- u RING-Komplex	*Hp* [58] *Pp* [59] *Sc* [60] *Yl* [61]
(Pex9p)	IMP		Matrixproteinimport; ORF falsch identifiziert; entspricht *Hs*Pex26p	*Yl* [62/63]
Pex10p	IMP	RING-Finger	Matrixproteinimport	*At* [64] *Hp* [65] *Hs* [66] *Pp* [67] *Sc* [68] *Tb* [69]
Pex11p	IMP MPG		Proliferation	*Cb* [70] *Hs* [71] *Pc* [72] *Rn* [73] *Sc* [74] *Tb* [75]
Pex12p	IMP	RING-Finger	Matrixproteinimport	*At* [76] *Hs* [77] *Pp* [78] *Rn*[79] *Sc* [80] *Tb* [81]
Pex13p	IMP	SH3	Matrixproteinimport; Rezeptor-Docking	*Cg* [82] *Hs* [83] *Pp* [84] *Sc* [85]
Pex14p	MGP, IMP	*Coiled coil*; PXXP	Matrixproteinimport Rezeptor-Docking	*At* [86] *Cl* [87] *Hp* [88] *Hs* [89] *Ld* [90] *Pp* [91] *Rn* [92] *Sc* [93]
Pex15p	IMP		Matrixproteinimport; verankert Pex1p/Pex6p-Komplex (*Sc)*	*Sc* [94]
Pex16p	MGP, IMP		Membranbiogenese; PMP Targeting	*At* [95] *Hs* [96] *Yl* [97]
Pex17p	MGP	*Coiled coil*	Matrixproteinimport	*Pp* [98] *Sc* [99]
Pex18p	LP, MGP		Matrixproteinimport; PTS2- abhängiger Proteinimport *(Sc)*	*Sc* [100]
Pex19p	LP MGP	CAAX-Box	Membranbiogenese; PMP Targeting	*At* [101] *Cg* [102] *Hp* [103] *Hs* [104] *Lm*[105] *Pp* [106] *Sc* [107] *Tb* [105]
Pex20p	MGP		Matrixproteinimport; PTS2-abhängiger Proteinimport	*Nc* [108] *Hp* [109] *Pp* [110] *Yl* [111]
Pex21p	LP MGP		Matrixproteinimport; PTS2- abhängiger Proteinimport *(Sc)*	*Sc* [112]
Pex22p	IMP		Matrixproteinimport; Pex4p-Verankerung	*At* [113] *Pp* [114] *Sc* [115]
Pex23p	IMP		Matrixproteinimport	*Yl* [116]
Pex24p	IMP		Matrixproteinimport	*Yl* [117]
Pex25p	MGP		Proliferation und Teilung	*Sc* [118]
Pex26p	IMP		Matrixproteinimport; verankert Pex1p/Pex6p-Komplex *(Hs)*	*Hs* [119]
Pex27p	MPG		Proliferation; Regulierung von Größe und Anzahl der Peroxisomen	*Sc* [118]
Pex28p	IMP		Proliferation; Regulierung von Größe und Anzahl der Peroxisomen	*Sc* [120]
Pex29p	IMP		Proliferation; Regulierung von Größe und Anzahl der Peroxisomen	*Sc* [120]
Pex30p	IMP		Proliferation; Regulierung von Größe und Anzahl der Peroxisomen	*Sc* [121]
Pex31p	IMP		Proliferation; Regulierung von Größe und Anzahl der Peroxisomen	*Sc* [121]
Pex32p	IMP		Proliferation Regulierung der Größe und Anzahl der Peroxisomen	*Sc* [121]

Organismus
***At**: Arabidopsis thaliana; **Ca**: Candida albicans; **Cb**: Candida boidinii; **Cg**: Cricetulus griseus; **Cl**: Cricetulus longicaudatus; **Cia**: Citrullus lanatus; **Cla**: Colletotrichum lagenarium; **Gh**: Gossypium hirsutum; **Ha**: Helianthus annuus; **Hp**: Hansenula polymorpha; **Hs:** Homo sapiens; **Kl**: Klyveromyces lactis ; **Ld**: Leishmania donovani; **Lm**: Leishmania donovani; **Mm**: Mus musculus; **Nc**: Neurospora crassa; **Nt**: Nicotiana tabacum; **Os**: Oryza sativa; **Pa**: Podospora anserina; **Pc**: Penicillium chrysogenum; **Pp**: Pichia pastoris; **Rn**: Rattus norvegicus; **Sc**: Saccharomyces cerevisiae; **Tb**: Trypanosoma brucei; **Yl**: Yarrowia lipolytica*

Lokalisation
LP: lösliches Protein; **IMP**: Integrales Membranprotein; **IP**: Intraperoxisomal; **MGP**: Membrangebundenes Protein

Strukturmotiv
AAA: **A**TPases **a**ssociated with diverse cellular **a**ctivites; **CAAX**: **C**=Cys-**A**=aliphatisch-**X**= Ser/Met; **Leucin Zipper**: $LX_6LX_6LX_6L$; **PXXP**: Klasse II Motiv für SH3-Liganden; **RING**: **r**eally **i**nteresting **n**ew **g**ene; **SH3**: **S**rc **h**omology **3**; **TPR**: **T**etratrico**p**eptide **r**epeat; **UBC**: **Ub**iquitin **c**onjugating enzyme; **WD40**: **40** Aminosäuren umfassendes Motiv, welches mit Tryp(**W**)/Asp(**D**) endet

Referenz
1. (Kiel *et al.*, 1999), **2.** (Portsteffen *et al.*, 1997), **3.** (Kiel *et al.*, 2000), **4.** (Heyman *et al.*, 1994), **5.** (Erdmann *et al.*, 1991), **6.** (Thieringer & Raetz, 1993), **7.** (Shimozawa *et al.*, 1992), **8.** (Flaspohler *et al.*, 1997), **9.** (Wilson & Bryant, 1994), **10.** (Berteaux-Lecellier *et al.*, 1995), **11.** (Waterham *et al.*, 1996), **12.** (Tsukamoto *et al.*, 1991), **13.** (Erdmann & Kunau, 1992), **14.** (Eitzen *et al.*, 1996), **15.** (Baerends *et al.*, 1996), **16.** (Kammerer *et al.*, 1998), **17.** (Wiemer *et al.*, 1996), **18.** (Höhfeld *et al.*, 1991), **19.** (Zolman *et al*., 2005), **20.** (van der Klei *et al.*, 1998), **21.** (Crane *et al.*, 1994), **22.** (Wiebel & Kunau, 1992), **23.** (Brickner & Olsen, 1998), **24.** (Piekarska *et al.*, 2006), **25.** (Wimmer *et al.*, 1998), **26.** (van der Klei *et al.*, 1995), **27.** (Dodt *et al.*, 1995), **28.** (Jardim *et al.*, 2000), **29.** (Baes *et al.*, 1997), **30.** (Kragler *et al.*, 1998), **31.** (Lee *et al.*, 2006), **32.** (Bonnet *et al.*, 2006), **33.** (Kiel *et al.*, 2004), **34.** (McCollum *et al.*, 1993), **35.** (van der Leij *et al.*, 1993), **36.** (De Walque *et al.*, 2000), **37.** (Szilard *et al.*, 1995), **38.** (Zolman & Bartel, 2004), **39.** (Kimura *et al.*, 2001), **40.** (Kaplan *et al.*, 2001), **41.** (Kiel *et al.*, 1999), **42.** (Yahraus *et al.*, 1996), **43.** (Kaplan *et al.*, 2001), **44.** (Kiel *et al.*, 2000), **45.** (Spong & Subramani, 1993), **46.** (Tsukamoto *et al.*, 1995), **47.** (Voorn-Brouwer *et al.*, 1993), **48.** (Krazy & Michels, 2005), **49.** (Nuttley *et al.*, 1994), **50.** (Schumann *et al.*, 1999), **51.** (Li *et al.*, 2003), **52.** (Braverman *et al.*, 1997), **53.** (Purdue *et al.*, 1997), **54.** (Braverman *et al.*, 1997), **55.** (Sichting *et al.*, 2003), **56.** (Elgersma *et al.*, 1998), **57.** (Marzioch *et al.*, 1994), **58.** (Waterham *et al.*, 1994), **59.** (Liu *et al.*, 1995), **60.** (Rehling *et al.*, 2000), **61.** (Smith *et al.*, 1997), **62.** (Eitzen *et al.*, 1995), **63.** (Kiel *et al*., 2006), **64.** (Schumann *et al.*, 1999), **65.** (Tan *et al.*, 1995), **66.** (Warren *et al.*, 1998), **67.** (Kalish *et al.*, 1995), **68.** (Erdmann & Kunau, 1992), **69.** (Krazy & Michels, 2005), **70.** (Marshall *et al.*, 1995),**71.** (Abe & Fujiki, 1998), **72.** (Kiel *et al*., 2005), **73.** (Passreiter *et al*., 1998), **74.** (Erdmann & Blobel, 1995), **75.** (Lorenz *et al*., 1998), **76.** (Fan *et al*., 2002), **77.** (Chang *et al.*, 1997), **78.** (Kalish *et al.*, 1996), **79.** (Okumoto *et al*., 1998), **80.** (Albertini *et al.*, 2001), **81.** (Krazy & Michels, 2005), **82.** (Toyama *et al.*, 1999), **83.** (Gould *et al.*, 1996), **84.** (Gould *et al.*, 1996), **85.** (Erdmann & Blobel, 1996), **86.** (Hayashi & Nito, 2000), **87.** (Shimizu *et al.*, 1999), **88.** (Komori *et al.*, 1997), **89.** (Fransen *et al.*, 1998), **90.** (Jardim *et al.*, 2002), **91.** (Johnson *et al.*, 2001), **92.** (Shimizu *et al.*, 1999), **93.** (Albertini *et al.*, 1997), **94.** (Elgersma *et al.*, 1997), **95.** (Lin *et al.*, 1999), **96.** (South & Gould, 1999), **97.** (Eitzen *et al.*, 1997), **98.** (Snyder *et al.*, 1999). **99.** (Huhse *et al.*, 1998), **100.** (Purdue *et al.*, 1998), **101.** (Hadden *et al.*, 2006), **102.** (James *et al.*, 1994), **103.** (Otzen *et al.*, 2004), **104.** (Fransen *et al.*, 2001), **105.** (Banerjee *et al.*, 2005), **106.** (Snyder *et al.*, 2000), **107.** (Götte *et al.*, 1998), **108.** (Sichting *et al.*, 2003), **109.** (Otzen *et al.*, 2005), **110.** (Leon *et al.*, 2006), **111.** (Titorenko *et al.*, 1998), **112.** (Purdue *et al.*, 1998), **113.** (Zolman *et al.*, 2005), **114.** (Koller *et al.*, 1999), **115.** (Koller *et al.*, 1999), **116.** (Brown *et al.*, 2000), **117.** (Tam & Rachubinski, 2002), **118.** (Rottensteiner *et al.*, 2003), **119.** (Matsumoto *et al.*, 2003), **120.** (Vizeacoumar *et al.*, 2003), **121.** (Vizeacoumar *et al.*, 2004)

1.4 Biogenese der peroxisomalen Membran

Es existieren unterschiedliche Vorstellungen über den genauen Ablauf der peroxisomalen Membranbiogenese. Frühe Studien nahmen aufgrund elektronenmikroskopischer Aufnahmen an, dass Peroxisomen durch Abknospung vom Endoplasmatischen Retikulum (ER) entstehen (Novikoff & Novikoff, 1972). Daneben existierten jedoch biochemische Daten, die demonstrierten, dass peroxisomale Matrixproteine an freien Ribosomen im Zytosol gebildet und posttranslational in Peroxisomen importiert werden (Rachubinski *et al.*, 1984). Aufgrund letzterer Beobachtungen wurde ein ER-unabhängiges Modell für die Biogenese der Peroxisomen entwickelt. Nach diesem „Wachstums- und Teilungsmodell" (*growth and divison-model*) werden alle peroxisomalen Matrix- und Membranproteine an freien Ribosomen synthetisiert und in bereits bestehende Peroxisomen importiert werden. Diese wachsen weiter an, um sich schließlich nach Erreichen einer kritischen Größe zu teilen (Lazarow & Fujiki, 1985). Für den Teilungsprozess wurde später eine Beteiligung von Pex11p beschrieben, da in einer *pex11*-Deletionsmutante häufig nur ein einzelnes großes Peroxisom in der Zellen existiert, welches nicht mehr zur Teilung fähig ist (Erdmann & Blobel; 1995; Marshall *et al.*, 1995). Eine Funktion in der Kontrolle der Größe und Vererbung von Peroxisiomen kommt den Pex11p-ähnlichen Peroxinen Pex25p und Pex27p zu (Rottensteiner *et al.*, 2003), wie auch den Peroxinen Pex30p, Pex31p und Pex32p (Vizeacoumar *et al.*, 2004). Auch dem Dynamin-ähnlichen Protein DLP1 aus Säugern (Hoepfner *et al.*, 2001), beziehungsweise Vps1p und Dnm1p aus *S. cerevisiae* (Koch *et al.*, 2003; Kuravi *et al.*, 2006) wird eine Rolle in der Regulation der Peroxisomenanzahl zugeschrieben. Die Peroxine Pex3p und Pex19p, sowie in manchen Organismen zusätzlich Pex16p sind für die grundsätzliche Bildung der peroxisomalen Membran durch den Import von peroxisomalen Membranproteinen (PMPs) essentiell (Eitzen *et al.*, 1997; South *et al.*, 1999; Hettema *et al.*, 2000). Pex19p interagiert mit zahlreichen peroxisomalen Membranproteinen und fungiert vermutlich als deren löslicher Rezeptor (Sacksteder *et al.*, 2000; Fransen *et al.*, 2001). Die komplex definierte, über eine Vorhersage-Matrix zu berechnende Konsensus-Sequenz der Pex19p-Bindestellen in PMPs ist für den korrekten Import der Membranproteine notwendig. Als zweiter essentieller Faktor wird eine Ankersequenz benötigt, welche bei integralen Membranproteinen der membrangängige Anteil darstellt (Rottensteiner *et al.*, 2004), während bei peripheren PMP`s die Bindestelle zu dem jeweiligen Ankerprotein benötigt wird (Girzalsky *et al.*, 2006). Gemeinsam konstituieren beide Faktoren das sog. mPTS (*membrane peroxisomal targeting signal*). Das mit PMPs beladene Pex19p bindet über das integrale Membranprotein Pex3p an die peroxisomale

Membran (Fang *et al*., 2004). Der Mechanismus der darauf folgenden Insertion der Membranproteine ist bisher jedoch noch nicht geklärt. Auf Grundlage der Gesamtheit der publizierten Daten zur Entstehung und Proliferation wurden Peroxisomen als autonome Organellen, wie Mitochondrien und Chloroplasten, angesehen. Jedoch blieb die Beobachtung, dass in bestimmten Mutanten, die niemals peroxisomale Strukturen enthalten haben, diese Organellen nach Einbringen des Wildtyp Gens *de novo* gebildet werden können, schwer zu erklären. In diesen Zusammenhang zeigen aktuelle Ergebnisse erneut eine Involvierung des ER auf. Mit Hilfe von fluoreszenzmikroskopischen Analysemethoden konnten verschiedene Arbeitsgruppen zeitaufgelöste Daten gewinnen, nach denen neu gebildetes Pex3p erst im ER anreichert wird, um dann in Pex3p enthaltenden Strukturen abhängig von Pex19p vom ER abgeschnürt zu werden, welche dann letztendlich zu importkompetenten Peroxisomen reifen (zur Übersicht Kunau, 2005; van der Zand *et al*., 2006). Somit sind Peroxisomen aller Wahrscheinlichkeit nach nicht endosymbiontischen Ursprungs, sondern eher als ein weitgehend selbstständiger Teil des Sekretionsweges anzusprechen (Schekman, 2005).

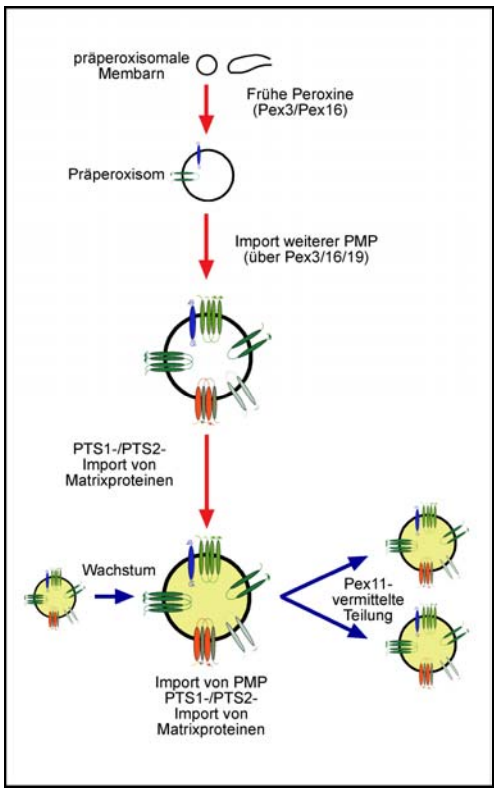

Abb.1.2: **Modell zur Biogenese der peroxisomalen Membran**. Der erste Weg (rot) beginnt mit einer sich vom ER ableitenden präperoxisomale Membran, die Pex3p bzw. Pex16p enthält. Durch Pex19p werden PMPs in die präperoxisomale Membran importiert. Danach kann der Import von Matrixproteinen erfolgen, womit die Reifung des Peroxisoms abgeschlossen ist. Der zweite Weg (blau) basiert auf dem „Wachstums- und Teilungsmodell“ nach dem sich neue Peroxisomen durch Teilung eines reifen Organells entwickeln. Die gelbe Füllung zeigt den vollständigen Satz von Matrixproteinen an. (aus Eckert & Erdmann, 2003, verändert)

1.5 Import peroxisomaler Matrixproteine

1.5.1 Prinzipien des Proteintransports in der Zelle

Der Transport von Proteinen über Membranen in eukaryotischen und prokaryotischen Systemen kann grundsätzlich anhand zweier zentraler Parameter charakterisiert werden: zum einen anhand der Natur der zu importierenden Kargo-Proteine sowie zum anderen über die Klassifizierung ihrer Rezeptoren. In Bezug auf den ersten Punkt besteht ein Kriterium darin, ob die zu transportierenden Proteine im ungefalteten oder im nativen Zustand vorliegen müssen. Beispiele für Systeme, welche ungefaltete Proteine transportieren, sind die SecYEG-verwandten Translokons der zytoplasmatischen Membran von Bakterien (Collinson, 2005), das ER (Osborne *et al.*, 2006), die Komplexe der äußeren (TOC) und inneren (TIC) Membran der Chloroplasten (Soll & Schleiff, 2004) sowie der äußeren (TOM) und inneren (TIM) Membran der Mitochondrien (Rehling *et al.*, 2004). Systeme für den Transport von gefalteten Proteinen translozieren oftmals auch Kofaktor-gebundene und oligomere Proteine. Hierzu ist der *Twin Arginine Translocator (Tat) Pathway* in der zytoplasmatischen Membran von Bakterien sowie in der Thylakoid Membran der Chloroplasten zu nennen (Muller & Klosgen, 2005), das Typ II Sekretionssystem in der äußeren Membran von Gram-negativen Bakterien (Russel, 1998), sowie der Proteintransport in den Nukleus (Pemberton & Paschal, 2005) und in Peroxisomen (Heiland & Erdmann, 2005).

Die zum Proteintransport dienenden Systeme können zusätzlich nach der Dynamik ihrer Rezeptoren in drei Kategorien unterteilt werden. Die Rezeptoren können (*i*) permanent membrangebunden vorliegen (*membrane-bound receptor*), wie es dies bei Tom20 und Tom70 des mitochondrialen TOM Komplexes (Taylor & Pfanner, 2004) sowie beim TatC Rezeptor des bakteriellen *Tat Pathways* der Fall ist (Muller & Klosgen, 2005). Das zweite Modell (*ii*) beinhaltet Faktoren zur Signalerkennung, die in löslichem Zustand ihr Kargo schon im Zytosol erkennen und zur Zielmembran dirigieren (*simple shuttle*). Dieser Mechanismus findet sich bei den *Signal Recognition Particel* (SRP) (Pool, 2005), bei SecA während der Sekretion von Protein in Prokaryoten (Karamyshev *et al.*, 2005) und vermutlich bei den Toc159 Rezeptoren der Chloroplasten (Gutensohn *et al.*, 2006). Die dritte Rezeptorklasse (*iii*) bindet ihr Kargo ebenfalls im Zytosol, dirigiert es zur Zielmembran und überquert als Rezeptor-Kargo-Komplex die Membran gänzlich. Nach Dissoziation des Kargos gelangt der Rezeptor in das Zytosol zurück (*extended shuttle*). Das *extended shuttle* findet sich bei den Importinen des Nukleus (Pemberton & Paschal, 2005), sowie bei den PTS-Rezeptoren des peroxisomalen Matrix Proteinimports (Heiland & Erdmann, 2005). Das Konzept des

Proteinimports in den Nukleus und in Peroxisomen hebt sich somit von dem anderer Organellen dadurch ab, dass gefaltete und oligomere Proteine mittels des *extended shuttle* importiert werden können. Das Prinzip des peroxisomalen Proteinimports unterscheidet sich von dem des Kerns darin, dass die peroxisomale Importmaschinerie induzierbar ist und dass ausschließlich ATP anstelle von GTP benötigt wird (zur Übersicht Wickner & Schekman, 2005).

Der Zyklus der peroxisomalen Importrezeptoren kann konzeptionell in vier Abschnitte untergliedert werden, welche im Folgenden näher beschrieben werden sollen: (*i*) Die Erkennung des Kargoproteins durch den Rezeptor im Zytosol, (*ii*) die Anbindung des Rezeptor-Kargo-Komplexes an die peroxisomale Membran, (*iii*) die Translokation des Komplexes über die Membran in das Lumen, einhergehend mit der Dissoziation des Kargos, sowie (*iv*) die Freisetzung des Rezeptors zurück ins Zytoplasma.

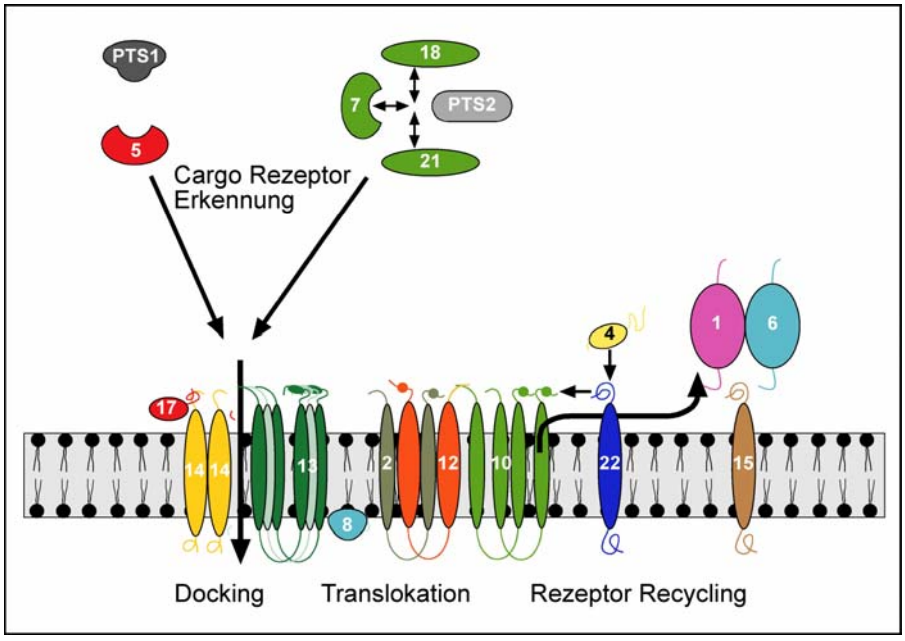

Abb.1.3.: Modell des peroxisomalen Matrixproteinimports. Proteine mit einem PTS1 oder PTS2 Signal werden im Zytosol von den spezifischen Rezeptoren erkannt, gebunden und als Rezeptor-Kargokomplex zur peroxisomalen Membran dirigiert. Danach folgt die Anbindung des Komplexes an die peroxisomalen Membranproteine. Nach der Translokation des Komplexes und Freisetzung der Kargoproteine ins peroxisomale Lumen schließt die Importkaskade mit dem Recycling des Rezeptors ins Zytosol (aus Eckert & Erdmann, 2003, verändert).

1.5.2 Peroxisomale Signalsequenz vermittelte Rezeptor-Substrat Bindung im Zytosol

Proteine, die für den Import in Peroxisomen bestimmt sind, gelangen auf einem von prinzipiell zwei Transportwegen an ihren Bestimmungsort. Determiniert werden diese über

zwei unterschiedliche Signalsequenzen. Die meisten peroxisomalen Matrixproteine besitzen ein PTS1 (*peroxisomal targeting signal type 1*)-Signal, welches sich am extremen Carboxy-Terminus befindet. Es besteht in der Regel aus dem Tripeptid „SKL“ oder einer spezies-spezifischen Variante, die sich dann von der allgemeinen Konsensussequenz (S/A/C)(K/R/H)(L/A) ableitet (Gould *et al.*, 1989; zur Übersicht Brocard & Hartig, 2006). Der Signalerkennungsfaktor Pex5p interagiert mit dem PTS1-Signal via sechs Tetratricopeptid-Sequenzen (TPR, *tetratricopeptide repeats*), die in dessen carboxy-terminaler Hälfte lokalisiert sind (McCollumn *et al.*, 1993) und fungiert somit als Importrezeptor. Das weitaus seltener vorkommende PTS2 ist ein Nonapeptid mit der Konsensussequenz (R/K)(L/V/I)X_5(H/Q)(L/A/F), welches im amino-terminalen Bereich des jeweiligen Proteins lokalisiert ist (Swinkels *et al.*, 1991). Die PTS2-enthaltenen Kargo-Proteine werden von dem WD40 Protein Pex7p erkannt (Marzioch *et al.*, 1994; Zhang *et al.*, 1995; zur Übersicht Lazarow, 2006). Gegenüber dem PTS1-Importweg besteht für den PTS2-Import eine größere Variation zwischen den Spezies. Das Extrem stellt *C. elegans* dar, dem der PTS2-Weg vollkommen fehlt (Motley *et al.*, 2000). Eine Besonderheit des PTS2-Importes ist die Existenz von notwendigen Kofaktoren des Rezeptors. In *S. cerevisiae* werden die funktionell redundanten Peroxine Pex18p und Pex21p für die Stabilisierung und Zielsteuerung des Kargo-Rezeptorkomplexes zu der peroxisomalen Membran benötigt (Purdue *et al.*, 1998; Stein *et al.*, 2002). Diese Funktion wird in einer Reihe weiterer Pilze und Hefen von Pex20p übernommen (vergleiche Tabelle 1.1; zur Übersicht Schliebs & Kunau, 2006). Beim Menschen ist durch einen Ausfall von PEX5 sowohl der PTS1- als auch der PTS2-Importweg betroffen. In Säugerzellen konnten eine kurze und eine lange Isoform von Pex5p identifiziert werden, die entsprechend als Pex5S (*small*) und Pex5L (*long*) bezeichnet werden. Diese Proteine unterscheiden sich nur durch eine kurze Insertion in Pex5L, welche in der Lage ist den PTS2-Rezeptor zu binden (Braverman *et al.*, 1998; Otera *et al.*, 2000). Zusätzlich konnte gezeigt werden, dass auch in Pflanzen der PTS2 Importwege abhängig vom PTS1-Rezeptor ist (Woodward & Bartel, 2005; Lee *et al.*, 2006). Während somit in Hefen und Pilzen beide PTS Importwege die gleiche membranständige Protein-Import-Maschinerie teilen, konvergieren sie in höheren Eukaryonten schon im Zytosol auf der Höhe von Pex5L.

Im peroxisomalen Lumen sind zudem einige Proteine lokalisiert, die kein PTS-Signal aufweisen. Der Import dieser Non-PTS Proteine erfolgt über verschiedene individuelle Mechanismen. So wird beispielsweise Acyl-CoA-Oxidase (Fox1p) in *S. cerevisiae* nicht an dem TPR-Bereich von Pex5p, sondern in dessen amino-terminaler Hälfte gebunden (Small *et al.*, 1988; Skoneczy & Lazarow, 1998; Klein *et al.*, 2002). Zudem existieren Hinweise, dass

Proteine, denen eine PTS-Sequenz fehlt, an Proteine binden können, die ein PTS-Signal aufweisen und „huckepack“ (*piggy-back*) zusammen mit diesen importiert werden. Dies wurde z.B. für die Enoyl-CoA-Isomerasen Dci1p und Eci1p aus *S. cerevisiae* (Yang *et al.*, 2001) demonstriert. Trotz dieser verschiedenen Wege verläuft der Non-PTS Import immer in Abhängigkeit von Pex5p (zur Übersicht van der Klei & Veenhuis, 2006).

1.5.3 Anbindung des Rezeptor-Kargo-Komplexes an die peroxisomale Membran

Die Peroxine Pex13p, Pex14p und Pex17p sind als Komponenten des sogenannten Docking-Komplexes beschrieben worden. Sowohl Pex14p als auch das integrale Pex13p interagieren miteinander wie auch mit den PTS-Rezeptoren (Albertini *et al.*, 1997; Erdmann & Blobel, 1996; Elgersma *et al.*, 1996; Gould *et al.*, 1996; zur Übersicht Eckert & Erdmann, 2003). Zentral für jeweils beide Interaktionen ist bei Pex14p das PXXP-Motiv (Bottger *et al.*, 2000) und bei Pex13p die SH3-Domäne (Pires *et al.*, 2003). Zudem weisen Pex14p wie auch Pex13p jeweils mehrere, z.T. in der Membran lokalisierte Interaktionsbereiche für Pex5p auf (Schell-Steven *et al.*, 2005; Williams *et al.*, 2005), was auf eine sehr dynamische Interaktionssequenz hindeutet. Weiterhin konnte demonstriert werden, dass Pex5p und Pex7p von jeweils distinkten Bereichen auf Pex13p bzw. Pex14p gebunden werden (Girzalsky *et al.*, 1999; Otera *et al.* 2002; Stein *et al.*, 2002; Niederhoff *et al.*, 2005), was ein Prinzip darstellen mag, das auch auf die anderen membranständigen Proteine der Importmaschinerie übertragbar sein könnte. Für Pex18p und Pex21p finden sich in der Literatur konträre Vorstellungen darüber, ob sie eine direkte (Einwächter *et al.*, 2001) oder eine indirekt über Pex7p vermittelte Interaktion mit den Docking-Komponenten eingehen können (Stein *et al.*, 2002). Pex20p scheint jedoch unabhängig von Pex7p mit Pex14p und Pex13p interagieren zu können (Einwächter *et al.*, 2001; Leon *et al.*, 2006). Pex17p ist über Pex14p mit der peroxisomalen Membran assoziiert (Huhse *et al.*, 1998; Girzalsky *et al*, 2006), weshalb es ebenfalls als Bestandteil des Docking-Komplexes angesehen wird. Da keine gesichert Pex14p-unabhängige Interaktion mit den PTS-Rezeptoren beschrieben wurde, ist die Funktion von Pex17p in diesem Zusammenhang noch ungeklärt. Es konnte demonstriert werden, dass Pex14p eine höhere Bindungsaffinität zu Kargo-beladenem Pex5p und Pex7p aufweist, während Pex13p eine höhere Affinität zu nicht beladenem Pex5p und Pex7p zeigt (Urquhart *et al.*, 2000; Otera *et al.*, 2000; Otera *et al.*, 2002). Auf Basis dieser Daten wird die Bindung von Pex14p als Eintritt von Pex5p in das komplexe Netzwerk von Protein-Protein-Interaktionen an der peroxisomalen Membran angesehen.

1.5.5 Translokation und Kargo-Dissoziation

Nach der Anbindung der PTS-Rezeptoren an den Docking-Komplex erfolgt als nächster Teilschritt der Importreaktion die Translokation des Rezeptor-Kargo-Komplexes in das peroxisomale Lumen. Weder der dieser Translokation zugrundeliegende Mechanismus, noch die Komponenten des Translokons konnten bisher identifiziert werden. In diesem Zusammenhang wird die Möglichkeit diskutiert, dass Komponenten des Docking-Komplexes selber einen Teil dieses hyothetischen Translokons darstellen könnten (Holroyd & Erdmann, 2001; Azevedo *et al.*, 2004).

Die vielen Bindemöglichkeiten von Pex5p an den membranständigen Peroxinen suggeriert, dass der PTS1-Rezeptor eine Kaskade von Wechselwirkungen während der Importreaktion durchläuft. Eine Besonderheit von Pex5p besteht darin, dass es während der Importreaktion seine Membrantopologie zu ändern scheint, da es sich nach der Bindung mit Pex14p wie ein stabil in die Membran integriertes Protein verhält (Gouveia *et al.*, 2000).

Letztendlich erreicht Pex5p das peroxisomale Lumen (Dodt & Gould, 1996; Dammai & Subramani, 2001), wobei jedoch noch ungeklärt ist, ob nur ein Teil von Pex5p (*simple shuttle hypothesis*) oder der gesamte Rezeptor (*extendet shuttle hypothesis*) luminal wird (Kunau, 2001). Für Pex7p konnte ebenfalls Evidenz gesammelt werden, dass es sich hierbei um einem zyklisierenden Rezeptor handelt (Marzioch *et al.*, 1994; Nair *et al.*, 2004). Der Mechanismus der Kargo-Ablösung ist noch ungeklärt. Das intraperoxisomale Pex8p, welches peripher an der Innenseite der peroxisomalen Membran von *S. cerevisiae* lokalisiert ist, wird oftmals in diesem funktionellen Zusammenhang diskutiert (van der Klei *et al.*, 1995). Eine Besonderheit von Pex8p besteht darin, dass es sowohl ein PTS1- als auch ein PTS2-Signal aufweist (Waterham *et al.*, 1994; Smith *et al.*, 1997). Die Frage, ob der Import von Pex8p in Abhängigkeit von den PTS-Signalsequenzen über die PTS-Rezeptoren verläuft (Zhang *et al.*, 2006) oder unabhängig von diesen erfolgt (Rehling *et al.*, 2000; Wang *et al.*, 2003) wird noch kontrovers diskutiert. Eine gesicherte Funktion für Pex8p liegt in der strukturellen Organisierung der Importmaschinerie, indem Pex8p eine Aufgabe bei der Verbindung des Docking- und RING-Finger-Komplexes zugewiesen wird (Agne *et al.*, 2003).

1.5.6 Recycling des Rezeptors

Die PTS-Rezeptoren Pex5p und Pex7p gelangen nach der Dissoziation ihrer Kargoproteine im Lumen des Peroxisoms wieder zurück zum Zytosol (Dammai & Subramani, 2001; Nair *et al.*, 2004). Das Durchlaufen mehrerer Importzyklen konnte für die PTS2-Ko-Rezeptoren noch nicht zweifelsfrei gezeigt werden. In dem Prozeß der Freisetzung der Rezeptoren scheinen die Peroxine Pex2p, Pex10p und Pex12p involviert zu sein. Diese Peroxine gehören zur Familie der RING-Fingerproteine (*really interesting new gene*; zur Übersicht Borden, 2000). Die RING-Domäne befindet sich am Carboxy-Terminus der drei Proteine, die auf der zytosolischen Seite der peroxisomalen Membran lokalisiert ist. Pex12p interagiert über diese Domäne sowohl mit Pex10p (Okumoto *et al.*, 2000), als auch mit Pex5p (Chang *et al.*, 1999). Mutationen in Pex10p oder Pex12p führen zu einer Anreicherung von Pex5p an der peroxisomalen Membran, weshalb für die beiden Peroxine eine Funktion nach der Anbindung des Rezeptors an den Dockingkomplex vorgeschlagen wurde (Gould & Valle, 2000). Darüber hinaus mag zumindest Pex12p eine weitere Funktion im Rezeptor Recycling übernehmen, da in humanen Zelllinien mit einer *pex12*-Mutation der Rezeptor Pex5p im peroxisomalen Lumen akkumuliert (Dodt & Gould, 1996). Es ist bislang unklar, ob die RING-Peroxine eine Funktion bei der Translokation haben oder aber bei dem Rezeptor Recycling, eine Beteiligung an beiden Prozessen wäre ebenso möglich (zur Übersicht Baker & Sparkes, 2005).

Aufgrund einer Epistasis-Analyse in *P. pastoris* wurde vier weiteren Peroxinen, nämlich Pex1p, Pex6p, Pex4p und Pex22p, eine Funktion nach dem Translokationsschritt zugewiesen (Collins *et al.*, 2000). Bei Deletion dieser Peroxine konnten in *P. pastoris* peroxisomale Strukturen beobachtet werden, die geringe Spuren von Matrixproteinen enthielten (Spong & Subramani, 1993; Heyman *et al.*, 1994; Koller *et al.*, 1999; Collins *et al.*, 2000). Bei Pex1p und Pex6p handelt es sich um Mitglieder der AAA-Familie (*ATPases associated with various cellular activities*) (Erdmann *et al.*, 1991; Voorn-Brouwer *et al.*, 1993; zur Übersicht Hanson & Whiteheart, 2005). Die beiden AAA-Peroxine interagieren miteinander an der Membran und im Zytosol (Faber *et al.*, 1998; Tamura *et al.*, 1998; Kiel *et al.*, 1999). Pex6p bindet mit seinem N-Terminus zudem direkt an das peroxisomale Membranprotein Pex15p in *S. cerevisiae* (Birschmann *et al.*, 2003) bzw. Pex26p im menschlichen System (Matsumoto *et al.*, 2003). Für AAA-Proteine im allgemeinen wird eine Beteiligung an einer Vielzahl von Prozessen beschrieben, wie zum Beispiel die Degradation von Proteinen und die Assemblierung und Dissoziation von Proteinkomplexen (zur Übersicht siehe Hanson & Whiteheart, 2005). Auch die AAA-Peroxine mögen verschieden Aufgaben erfüllen. So deutete die erwähnte Epistasis-Analyse (Collins *et al.*, 2000) an, dass Pex1p und Pex6p eine

späte Aufgabe im Pex5p Zyklus zukommt und somit eventuell an der Freisetzung des Rezeptors beteiligt sind. Diese Vorstellung wurde jedoch von der gleichen Arbeitsgruppe in einem Hypothesen-Artikel (Gould & Collins, 2002) wieder abgeändert und eine Rolle bei der Disassemblierung von großen Rezeptor-Kargo-Komplexen vor der Translokation ins Innere des Peroxisoms postuliert. Davon unabhängig werden die AAA-Peroxine mit der Fusion peroxisomaler Vesikel in Verbindung gebracht (Titorenko *et al.*, 2000; Titorenko & Rachubinski, 2000).

Pex4p gehört zur Familie der Ubiquitin-konjugierenden Enzyme und ist über das integrale Peroxin Pex22p an der zytoplasmatischen Seite der peroxisomalen Membran lokalisiert (Wiebel & Kunau, 1992; Koller *et al*., 1999). Für Pex4p und Pex22p wird eine Funktion nach den AAA-Peroxinen Pex1p und Pex6p vermutet (Collins *et al*., 2000).

Die Beteiligung von einem Ubiquitin-konjugierenden Enzym (Ubc) an der peroxisomalen Biogenese deutet auf eine funktionelle Rolle von Ubiquitinylierungsprozessen beim peroxisomalen Matrixproteinimport hin. Somit ist es möglich, dass entweder Proteine der Importmaschinerie oder aber die PTS-Rezeptoren selber während der Importreaktion posttranslational mit Ubiquitin modifiziert werden. Die Identität der Zielproteine für diese Modifikation ist jedoch unbekannt. Pex4p ist zudem das einzige Ubc, welches essentiell für die Biogenese eines Organells ist.

1.6 Posttranslationale Modifikationen von Peroxinen

Das Konzept der zyklisierenden Rezeptoren impliziert, dass Pex5p und Pex7p eine sequentielle Abfolge von Interaktionen mit Proteinen der peroxisomalen Importmaschinerie eingehen und somit in definierter zeitlicher Abfolge Bestandteil verschiedener Proteinkomplexe werden. Eine Möglichkeit Protein-Protein Interaktionen oder die Zusammensetzung von Protein-Komplexen zu regulieren besteht in der spezifischen und reversiblen Markierung bestimmter Komponenten über posttranslationale Modifikationen (Nooren & Thornton, 2003). Zu Beginn dieser Arbeit war die kovalente Modifizierung von vier Peroxinen bekannt. Der lösliche PMP-Rezeptor Pex19p wird an der carboxy-terminal gelegenen *CAAX*-Box von der Farnesyltransferase prenyliert (Götte *et al*., 1998; Kammerer *et al*., 1997; Matsuzono *et al*., 1999). Die Funktion der Farnesylierung von Pex19p wird kontrovers diskutiert, da sie nicht essentiell für die peroxisomale Biogenese zu sein scheint (Vastiau *et al*., 2006). Pex14p, das periphere PTS-Rezeptor Docking-Protein, liegt teilweise in phosphorylierter Form vor (Komori *et al*., 1999; Johnson *et al*., 2001), was ebenfalls für das integrale Membranprotein Pex15p beschrieben wurde (Elgersma *et al*., 1997). Über die

Bedeutung und Funktion der Phosphorylierung sowie über die involvierten Kinasen ist nichts bekannt.

Der lösliche PTS2-Ko-Rezeptor Pex18p wird ubiquitinyliert (Purdue & Lazarow, 2001). Ubiquitin ist ein in allen Eukaryonten konserviertes Signalprotein, welches kovalent an das Zielprotein gebunden wird und auf diese Weise dessen Funktion oder Transport verändert. Die Modifikation wird durch den Ablauf einer hierarchisch gegliederten Enzymkaskade ermöglicht (Abb. 1.4), zu deren Beginn das Ubiquitin-Aktivierende Enzym (Uba1; E1) steht, welches unter ATP-Verbrauch das freie Ubiquitin als Thioester bindet. Das E1 überträgt in einer Transthiolierungsreaktion das aktivierte Ubiquitin auf den Cystein-Rest eines Ubiquitin-Konjugierenden Enzyms (Ubc; E2). Eine der zahlreichen Ubiquitin-Protein-Isopetid-Ligasen (E3) assoziiert zugleich mit dem Ubiquitin-beladenen E2 und dem Substrat. Die Funktion der E3 Enzyme determiniert die Substratspezifität der Reaktion, indem sie als eine Art Plattform fungieren, die E2 und Substrat stabil zusammenführt, um somit die Katalyse der Isopeptid-Bindung von Ubiquitin an ein Substrat zu ermöglichen. Ubiquitin kann als einzelnes Molekül oder aber als Ubiquitin-Kette an ein Zielprotein gebunden werden. Je nachdem, wie viele einzelne Ubiquitin-Moleküle an ein Zielprotein angebracht werden, oder welchen Form die Ubiquitin-Kette besitzt, wird das Zielprotein zu unterschiedlichen Zielen dirigiert (zur Übersicht Weissman, 2001; Hicke *et al*., 2005).

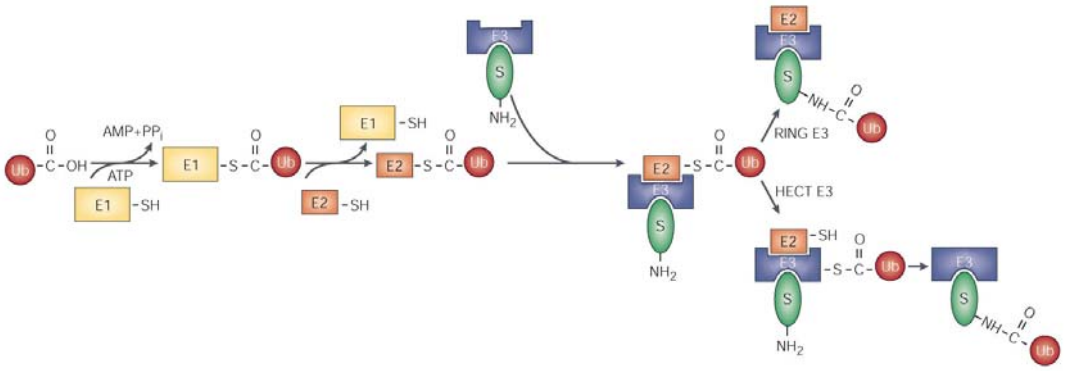

Abb. 1.4 Ubiquitinylierungs-Kaskade. Freies Ubiquitin wird unter ATP-Verbrauch durch eine Thiol-Ester-Bindung an das E1 gebunden und auf E2-Enzyme übertragen. Die Verbindung von Substrat und Ubiquitin wird von E3-Enzymen katalysiert, `wobei die Übertragung von Ubiquitin im Falle der HECT E3-Enzyme über ein E3-Ub-Intermediat verläuft, während RING E3 Enzyme eine direkte Übertragung des Ubiquitins katalysieren (aus Weissman, 2001)

Die bekannteste Funktion von Ubiquitinylierungsprozessen liegt in der Markierung von Proteinen, die zur Degradation bestimmt sind. Daher wird die Ubiquitinylierung von Pex18p in Zusammenhang mit dessen kurzer Lebensdauer gebracht, die mit einer Halbwertszeit von

10 Minuten angegeben wird (Purdue & Lazarow, 2001). Die Stabilität von Pex18p wird bei einer Doppel-Deletion der Ubiquitin-Konjugierenden Enzyme Ubc4p und Ubc5p, welche in viele Degradationsprozesse involviert sind, erhöht. Interessanterweise erfolgt eine deutliche Stabilisierung von Pex18p in Mutantenstämmen, in denen Komponenten der membrangebundenen peroxisomalen Matrix-Protein Importmaschinerie fehlen. Diese Beobachtung impliziert eine Verbindung zwischen einem – immer noch hypothetischen – kompletten Durchlaufen der Importmaschinerie von Pex18p und dessen Ubiquitinylierung und Degradation.

1.7 Zielsetzung dieser Arbeit

Nach dem Modell des PTS1-Rezeptorzyklus erkennt Pex5p die neusynthetisierten peroxisomalen Matrixproteine im Zytosol und dirigiert sie zur peroxisomalen Membran. Nach der Freisetzung des z.T. oligomeren Kargos im Lumen des Peroxisoms wird der Rezeptor wieder ins Zytosol freigesetzt. Da der peroxisomale Proteinimport somit untrennbar mit dem Rezeptorzyklus von Pex5p gekoppelt vorliegt, ist zur mechanistischen Entschlüsselung des Imports eine detaillierte Analyse des PTS1-Rezeptorzyklus essentiell.

In der vorliegenden Arbeit sollte untersucht werden, welche Faktoren den PTS1-Rezeptorzyklus in *S. cerevisiae* regulieren. Dabei sollte das Gewicht auf den späten Schritten liegen. In diesem Zusammenhang sollte zunächst analysiert werden, ob Pex5p posttranslational mit dem Signalprotein Ubiquitin modifiziert wird. Eine Präparationsmethode zur Darstellung des modifizierten Pex5p sollte etabliert werden, welche beispielsweise Inhibierung von deubiquitinylierenden Enzymen oder des Proteasoms durch chemische Inhibitoren oder konditionierte Mutanten beinhalten könnte. Die Form der Ubiquitinylierung sollte festgestellt werden, um mit Hilfe der Überexpression von verschiedenen Ubiquitin-Mutanten die Natur der *in vivo* Modifikation zu klären, also ob es sich um Mono- oder eine bestimmte Form der Polyubiquitinylierung handelt. Der Ort des Ubiquitinylierungsereignisses soll mittels Sedimentationsanalysen bestimmt werden. Das verantwortliche Ubiquitin-konjugierende Enzyme sollte durch Erstellung und Analyse von Deletionsstämmen identifiziert werden Um die Lysin-Reste von Pex5p, an welche das Ubiquitin angeheftet wird, zu identifizieren, sollen mittels gerichteter Mutagenese Punktmutanten erstellt und analysiert werden. Die Funktion der Ubiquitinylierung für den Pex5p Rezeptor-Zyklus soll letztendlich auf Grundlage der geplanten Experimente bestimmt und charakterisiert werden. In diesem Zusammenhang ist ein *in vitro* System für den Pex5p-Zyklus zu etablieren, um die gefundenen Parameter funktionell testen zu können.

2. ORIGINALARBEITEN

2.1

Ubiquitination of the peroxisomal import receptor Pex5p

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Ubiquitination of the peroxisomal import receptor Pex5p

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Proteins harbouring a peroxisomal targeting signal of type 1 (PTS1) are recognized by the import receptor Pex5p in the cytosol which directs them to a docking and translocation complex at the peroxisomal membrane. We demonstrate the ubiquitination of Pex5p in cells lacking components of the peroxisomal AAA (ATPases associated with various cellular activities) or Pex4p–Pex22p complexes of the peroxisomal protein import machinery and in cells affected in proteasomal degradation. In cells lacking components of the Pex4p–Pex22p complex, mono-ubiquitinated Pex5p represents the major modification, while in cells lacking components of the AAA complex polyubiquitinated forms are most prominent. Ubiquitination of Pex5p is shown to take place exclusively at the peroxisomal membrane after the docking step, and requires the presence of the RING-finger peroxin Pex10p. Mono- and poly-ubiquitination are demonstrated to depend on the ubiquitin-conjugating enzyme Ubc4p, suggesting that the ubiquitinated forms of Pex5p are targeted for proteasomal degradation. Accumulation of ubiquitinated Pex5p in proteasomal mutants demonstrates that the ubiquitination of Pex5p also takes place in strains which are not affected in peroxisomal biogenesis, indicating that the ubiquitination of Pex5p represents a genuine stage in the Pex5p receptor cycle.

Key words: Pex5p, peroxin, peroxisome, protein import, ubiquitination.

INTRODUCTION

Peroxisomes are membrane-bound organelles that play important roles in lipid metabolism in virtually all eukaryotes (reviewed in [1]). So far, 32 *PEX* genes have been demonstrated to be indispensable for peroxisome biogenesis. Most of their gene products, collectively named peroxins, are required for protein transport across the peroxisomal membrane from the cytoplasm into the peroxisomal matrix (reviewed in [2–4]). Two well-characterized peroxisomal targeting signals, PTS1 and PTS2, and the corresponding import receptors Pex5p and Pex7p, have been identified (reviewed in [5,6]). Pex5p consists of a C-terminal domain of six TPRs (tetratricopeptide repeats), which provides the binding site for the tripeptide PTS1. The PTS2 receptor is characterized by multiple WD40 repeats. Both receptors are predominantly localized in the cytosol, with a minor portion being associated with the peroxisomal membrane. The receptors are supposed to bind their cargo proteins in the cytosol and target them to the peroxisomal membrane. Whereas Pex5p is able to perform its role in PTS1 protein targeting on its own, the PTS2 receptor Pex7p needs the auxiliary proteins Pex18p and Pex21p [7]. Three peroxins, Pex13p, Pex14p and Pex17p, have been demonstrated to contribute to the docking of the receptors to the *cis*-side of the peroxisomal membrane [8–15]. According to the model of shuttling receptors [16], the import receptors release their cargo at the membrane or into the peroxisomal lumen and cycle back to the cytosol [16,17].

Downstream of the docking complex, a second complex comprising three RING-finger peroxins, Pex2p, Pex10p and Pex12p [18], has been implicated in cargo translocation [5,19]. Most interestingly, peroxisomes accommodate folded, even oligomeric proteins; however, the mechanism of how these proteins traverse the membrane is still unknown. The peroxins Pex1p and Pex6p are AAAs (ATPases associated with various cellular activities), which are required for matrix protein import and which are likely to be responsible for the overall ATP-dependence of the import process [20–23]. Pex4p and Pex22p have been proposed to act at later steps of the import process [18]. Pex4p, also referred to as Ubc10p, is anchored to the peroxisomal membrane by Pex22p and belongs to the Ub (ubiquitin)-conjugating enzyme family [24,25]. Even though the target of Pex4p has not been identified, Pex18p, which is involved in the peroxisomal import of PTS2 proteins, has been demonstrated to be constitutively degraded in a Ub-dependent manner [26].

In the present paper, we demonstrate the Ubc4p-dependent mono- and poly-ubiquitination of the PTS1 receptor Pex5p at the peroxisomal membrane of cells lacking components of the peroxisomal AAA or Pex4p–Pex22p complexes, or of proteasomal mutants. The ubiquitination of Pex5p takes place after the docking event and requires the presence of the RING-finger peroxin Pex10p. We also discuss the functional relevance of the ubiquitination of Pex5p for peroxisome biogenesis.

EXPERIMENTAL

Strains and culture conditions

Saccharomyces cerevisiae strains used in the present study are listed in Table 1. Deletion strains were generated by the 'short flanking homology' method using the removable *loxP-kanMX4-loxP* marker as described in [27]. Yeast complete (YPD) and minimal media (SD) have been described previously [28]. YNO medium contained 0.1 % (w/v) oleic acid, 0.05 % (v/v) Tween 40, 0.1 % (w/v) yeast extract and 0.67 % (w/v) yeast nitrogen base without amino acids, adjusted to pH 6.0. When necessary, auxotrophic requirements were added according to [29]. For induction of the *CUP1* promoter, $CuSO_4$ was added according to [16].

Abbreviations used: AAA, ATPases associated with various cellular activities; CIM, co-lethal in mitogenesis; GFP, green fluorescent protein; PTS, peroxisomal targeting signal; Ub, ubiquitin.

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Table 1 Yeast strains used in the present study

Strain	Description	Source of reference	Oligonucleotides
UTL-7a	MATa, ura3-52, trp1, leu2-3/112	[28]	
*pex1*Δ	*pex1*::loxP	Present study	KU681/KU682
*pex1*Δ *pex4*Δ	*pex1*::loxP, *pex4*::*kanMX4*	Present study	KU394/KU395
*pex1*Δ *ubc1*Δ	*pex1*::loxP, *ubc1*::CreloxP-*kanMX4*	Present study	KU990/KU991
*pex1*Δ *ubc2*Δ	*pex1*::*loxP*, *ubc2*::CreloxP-*kanMX4*	Present study	KU993/KU994
*pex1*Δ *ubc4*Δ	*pex1*::*loxP*, *ubc4*::CreloxP-*kanMX4*	Present study	KU996/KU997
*pex1*Δ *ubc5*Δ	*pex1*::*loxP*, *ubc5*::CreloxP-*kanMX4*	Present study	KU999/KU1000
*pex1*Δ *ubc6*Δ	*pex1*::*loxP*, *ubc6*::CreloxP-*kanMX4*	Present study	KU938/KU939
*pex1*Δ *ubc7*Δ	*pex1*::*loxP*, *ubc7*::CreloxP-*kanMX4*	Present study	KU941/KU942
*pex1*Δ *ubc8*Δ	*pex1*:*loxP*, *ubc8*::CreloxP-*kanMX4*	Present study	KU944/KU945
*pex1*Δ *ubc11*Δ	*pex1*::*loxP*, *ubc11*::CreloxP-*kanMX4*	Present study	KU947/KU948
*pex1*Δ *ubc12*Δ	*pex1*::*loxP*, *ubc12*::CreloxP-*kanMX4*	Present study	KU917/KU918
*pex1*Δ *ubc13*Δ	*pex1*::*loxP*, *ubc13*::CreloxP-*kanMX4*	Present study	KU1002/KU1003
*pex4*Δ	*pex4*::*LEU2*	[24]	
*pex4*Δ *ubc1*Δ	*pex4*::*LEU2*, *ubc1*::CreloxP-*kanMX4*	Present study	KU990/KU991
*pex4*Δ *ubc2*Δ	*pex4*::*LEU2*, *ubc2*::CreloxP-*kanMX4*	Present study	KU993/KU994
*pex4*Δ *ubc4*Δ	*pex4*::*LEU2*, *ubc4*::CreloxP-*kanMX4*	Present study	KU996/KU997
*pex4*Δ *ubc5*Δ	*pex4*::*LEU2*, *ubc5*::CreloxP-*kanMX4*	Present study	KU999/KU1000
*pex4*Δ *ubc6*Δ	*pex4*::*LEU2*, *ubc6*::CreloxP-*kanMX4*	Present study	KU938/KU939
*pex4*Δ *ubc7*Δ	*pex4*::*LEU2*, *ubc7*::CreloxP-*kanMX4*	Present study	KU941/KU942
*pex4*Δ *ubc8*Δ	*pex4*::*LEU2*, *ubc8*::CreloxP-*kanMX4*	Present study	KU944/KU945
*pex4*Δ *ubc11*Δ	*pex4*::*LEU2*, *ubc11*::CreloxP-*kanMX4*	Present study	KU947/KU948
*pex4*Δ *ubc12*Δ	*pex4*::*LEU2*, *ubc12*::CreloxP-*kanMX4*	Present study	KU917/KU918
*pex4*Δ *ubc13*Δ	*pex4*::*LEU2*, *ubc13*::CreloxP-*kanMX4*	Present study	KU1002/KU1003
*pex5*Δ	*pex5*::*kanMX4*	[35]	
*pex6*Δ	*pex6*::*LEU2*	[58]	
*pex10*Δ	*pex10*::*kanMX4*	[43]	
*pex13*Δ	*pex13*::URA3	[35]	
*pex15*Δ	*pex15*::LEU2	[58]	
*pex4*Δ*pex10*Δ	*pex4*::*kanMX4*, *pex10*::CreloxP-*kanMX4*	Present study	KU562/KU699
cim5-1	*cim5-1*, *ura3-52*, *leu2Δ1*, *his3Δ200*	[53]	
*cim5-1pex5*Δ	*pex5*::CreloxP-*kanMX4*, *cim5-1*	Present study	KU301/KU302
*cim5-1pex1*Δ	*pex1*::CreloxP-*kanMX4*, *cim5-1*	Present study	KU681/KU682

Oligonucleotides and plasmids

Oligonucleotides used are listed in Table 2. Ub and *myc*Ub were expressed under the control of the copper-inducible *CUP1* promoter [30] from plasmid YEp96 and YEp105 [31] respectively. For expression of Ub-K48R plasmid YEp110 was used [32].

Yeast cell extracts

Yeast cells were grown on 0.3 % SD medium to late exponential phase and subsequently for 15 h in YNOD [0.1 % (w/v) dextrose, 0.1 % (w/v) oleic acid, 0.05 % (v/v) Tween 40, 0.1 % (w/v) yeast extract and 0.67 % (w/v) yeast nitrogen base]. Cells were harvested and aliquots of 30 mg of cells were resuspended in 300 μl of potassium phosphate buffer (pH 7.4) containing 20 % trichloroacetic acid. The samples were frozen at -80 °C for at least 30 min. Samples were sedimented, washed twice with ice-cold 50 % acetone and resuspended in 80 μl of 10 % (w/v) SDS/0.1 M NaOH and 20 μl of SDS loading buffer [5 % (w/v) 2-mercaptoethanol, 15 % (v/v) glycerol and 0.01 % (w/v) Bromophenol Blue].

Membrane sedimentation

Oleic-acid-induced cells were washed with water and 1 g was used per sedimentation. A volume of 3 ml of buffer A (0.2 M Hepes, 1 M potassium acetate and 50 mM magnesium acetate, pH 7.5), protease inhibitors (8 μM antipain, 0.3 μM aprotinin, 1 μM bestatin, 10 μM chymostatin, 5 μM leupeptin, 1.5 μM pepstatin, 1 mM benzamidine and 1 mM PMSF; Boehringer Mannheim), 5 mM NaF and 3 g of glass beads (0.5 mm) was added to the cells. Breakage was achieved by vortex-mixing for 12 min (twelve 60 s bursts with breaks of at least 60 s on ice) [33]. Samples were transferred to Corex tubes and were centrifuged at 1500 ***g*** for 10 min. Supernatants were normalized for protein and volume, and membranes were sedimented at 40 850 rev./min for

Table 2 Oligonucleotides used in the present study

Oligonucleotide	Sequence
KU0394	5′-TGGAGACAACATAAAATATACATAATCATCGCCTTTATACATAATCGTACGCTGCAGGTCGAC-3′
KU0395	5′-GAGGGCCCATTGTTTGCCATTCGAACACATCCATCCTACGTGGTAATCGATGAATTCGAGCTCG-3′
KU0562	5′-GAGGGCGAAGTAGGTATTAGCCGTTTACATTAGAAAATAAGGTAGCGTACGCTGCAGGTCGAC-3′
KU0681	5′-GGACGGCAGTAACAAGAAACACCTGAGGAACTGCTCTTTCAACAGCTGAAGCTTCGTACGCT-3′
KU0682	5′-CAGCCGCATTTTTTGCCCTTTAAAGGGAAACGCGCTCCGTTCATAGGCCACCAGTGGATCTG-3′
KU0699	5′-GGCCTGTGGACAATGCTAAAAGAGTAGTCAAATTATTGACCAATAGGCCAGTAGTGGATCTG-3′
KU0990	5′-AGTCAATTGAAGCAAGTGTGACGACATAAGTATCGTAATTCAGCTGAAGCTTCGTACGCT-3′
KU0991	5′-TGATCATCAAGAACACGGATGCACAATGAAACATCCTCGCATAGGCCACTAGTGGATCTG-3′
KU0993	5′-ACTATCAAGTTGCAGAGTTGATGACATAAATAGTAGAGTGCAGCTGAAGCTTCGTACGCT-3′
KU0994	5′-GGTAATCGAATTCATAATATCGGCTCGGCATTCATCATTAATAATAGGCCACTAGTGGATCTG-3′
KU0996	5′-ATTTCACTGACTATAGAGTACATACATAAACAAGCATCCACAGCTGAABCTTCGTACGCT-3′
KU0997	5′-ACTGAAATATATGCGGGATATACCCGCGCTTGCGTGCATGATAGGCCACTAGTGGATCTG-3′
KU0999	5′-TCCAAGGTCAGGACTGCTTATTGACTACCATCTTGAAAAGCAGCTGAAGCTTCGTACGCT-3′
KU1000	5′-GCGCTGAGGAAGGTAAGTCTACACAATTTATCCGTTAGCCATAGGCCACTAGTGGATCTG-3′
KU0938	5′-TAGGAGCCGTGATAAAGAAGACTACCATCGCATATCGCAGCAGCTGAAGCTTCGTACGCT-3′
KU0939	5′-TGTCTATATAATACTATTGATTTCTATTCTGTGTTGTCAAATAGGCCACTAGTGGATCTG-3′
KU0941	5′-GGAACTTCCCTAGTAATAGTGTAATTTGGAAGGGCATAGCCAGCTGAAGCTTCGTACGCT-3′
KU0942	5′-GTTAAAAGGAAGACCAAATGATCATTAACCTGCTACCTGCATAGGCCACTAGTGGATCTG-3′
KU0944	5′-GTGAGCACCGGAAACAGCTATCTGCGCTTAAGTATCACTACAGCTGAAGCTTCGTACGCT-3′
KU0945	5′-TTGTAATTATAGTCGTACGTGTGGTATAGTATGCCATCATATAGGCCACTAGTGGATCTG-3′
KU0947	5′-AGGTGCTACAAAACCTGTATGATTCGAGGCTTGCTAGTATCAGCTGAAGCTTCGTACGCT-3′
KU0948	5′-CGCCGTAGAAAAATAGCATTGGAGTTTGAGAGTCTGTGGCATAGGCCACTAGTGGATCTG-3′
KU0917	5′-GTACGTAGATGCAACTATATCTTTCCGTTTCTCATTGGCGGTCAGCTGAAGCTTCGTACGCT-3′
KU0918	5′-AGCAAGATGTTACAAGGCGACAACTCGCCTAGTATGTCATAGGCCACTAGTGGATCTG-3′
KU1002	5′-GGATAAGTGATTCATTGAAAGATGGCGCGCCACCACGAACCAGCTGAAGCTTCGTACGCT-3′
KU1003	5′-ACATTAGAGTAGGACGGTCGATCCCGGCTTCCATGGAACGATAGGCCACTAGTGGATCTG-3′

30 min in a Sorvall AH650 rotor. The resulting pellet was resuspended in buffer A corresponding to the volume of the supernatant. Aliquots of the samples were analysed by SDS/PAGE.

Immunoblotting

Immunoblot analysis was performed according to standard protocols [34]. Immunoreactive complexes were visualized using horseradish-peroxidase-conjugated anti-rabbit or anti-mouse IgG in combination with the ECL® (enhanced chemiluminescence) system from Amersham Biosciences (Uppsala, Sweden). Polyclonal rabbit antibodies were against Pex5p [35], Pex13p [35] and fructose-1,6-bisphosphatase [36].

Microscopy

Analysis of live cells for GFP (green fluorescent protein) fluorescence was performed with a Zeiss Axioplan microscope and AxioVision 4.1 software (Zeiss, Jena, Germany). Before inspection, cells were grown for 2 days on solid minimal medium containing oleic acid as a sole carbon source.

Cell fractionation

Spheroplasting of yeast cells, homogenization and differential centrifugation at 25000 ***g*** of homogenates were performed as described previously [28].

RESULTS

Pex5p is modified in specific *pex* mutants

Previous studies have demonstrated that the steady-state concentration of Pex5p is influenced by the presence or absence of certain peroxins. Defects in either one of the human AAA peroxins Pex1p or Pex6p, as well as the Ub-conjugation enzyme Pex4p or its membrane anchor protein Pex22p of *Pichia pastoris*, resulted in a massive decrease of Pex5p level in these cells [17,18,37,38]. In order to analyse these observations in *S. cerevisiae*, we examined the abundance of Pex5p in 27 null mutant strains, each deleted for one of the known peroxins. Wild-type and mutant strains were cultured on oleic acid medium, and whole-cell lysates were prepared and analysed for the presence of Pex5p by immunoblotting. Although *pex* mutants cannot grow on oleic acid medium, induction of β-oxidation and proliferation of peroxisomes still takes place. In contrast with the above-mentioned observations for human and *P. pastoris* cells, the Pex5p amount within the different mutant strains of *S. cerevisiae* was not reduced, and Pex5p was indistinguishable from the wild-type protein in its size (results not shown). However, we noted that in *pex1*Δ, *pex6*Δ, *pex15*Δ, *pex4*Δ and *pex22*Δ mutant strains, additional higher-molecular-mass forms of Pex5p were labelled with the anti-Pex5p serum (Figure 1). Most interestingly, the mutants could be divided into two groups with respect to the Pex5p-modification pattern. The first group comprises the *pex1*Δ, *pex6*Δ and *pex15*Δ mutants, which were characterized by the presence of three additional protein bands for Pex5p. Group II consists of mutant *pex4*Δ and *pex22*Δ, which exhibit two additional bands, the higher one with the same migration behaviour as one of the bands of group I (Figure 1).

Pex5p is ubiquitinated in *pex1*Δ and *pex4*Δ mutant cells

The observed ubiquitination of Pex18p in yeast and the rapid degradation of Pex5p in other organisms tempted us to analyse whether the higher-molecular-mass species of Pex5p form by ubi-

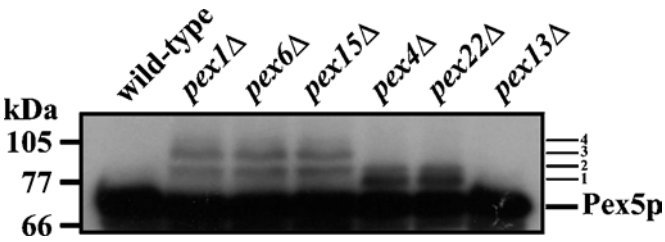


Figure 1 Pex5p-modification in *pex* mutants

Equal amounts of whole-cell trichloroacetic acid lysates of oleic-acid-induced wild-type, *pex1*Δ, *pex6*Δ, *pex15*Δ, *pex4*Δ, *pex22*Δ and *pex13*Δ cells were separated by SDS/PAGE and blotted on to a nitrocellulose filter. The filter was probed for the presence of Pex5p, and immunoreactive complexes were visualized with the ECL® system. Additional higher-molecular-mass forms of Pex5p are labelled 1–4.

quitination of the protein. In support of this assumption, the higher-molecular-mass species could be labelled with anti-Ub serum by immunoblot analysis (results not shown). For the ultimate proof of Pex5p ubiquitination, we also analysed yeast strains expressing either the wild-type Ub or the *myc*Ub fusion gene under the control of the yeast copper metallothionein (*CUP1*) promoter [31]. The epitope tag consists of a ten-residue sequence encoding a portion of the c-*myc* proto-oncogene product recognized by the monoclonal antibody 9E10 [39]. The *myc*Ub variant is about 1.5 kDa larger than wild-type Ub, but is indistinguishable from wild-type Ub in its ability to be enzymically conjugated to and cleaved from acceptor molecules [31]. If the high-molecular-mass species of Pex5p were Ub conjugates, *in vivo* substitution of the Ub with the larger *myc*Ub should lead to a decrease in electrophoretic mobility of these species. Wild-type, as well as *pex1*Δ and *pex4*Δ strains expressing either Ub or *myc*Ub, were grown on oleic acid medium with $CuSO_4$, which leads to an expression of plasmid-encoded Ub and *myc*Ub of up to 50–100 times greater than the endogenously produced levels of Ub [30]. Whole-cell lysates were prepared, and equal amounts of protein from each strain were separated by SDS/PAGE and processed for immunoblotting. Samples were probed with anti-Pex5p antibodies and slower migrating Pex5p species appeared (Figure 2). Comparison of samples from *pex1*Δ and *pex4*Δ expressing either Ub or *myc*Ub showed that the putative ubiquitinated species of Pex5p were replaced by slightly larger new bands. These data showed that overexpression of *myc*Ub was accompanied by an increase in size of all higher-molecular-mass Pex5p-species. Thus our data clearly demonstrate that Pex5p is ubiquitinated *in vivo*. In the following, the ubiquitinated Pex5p is referred to as Ub-Pex5p.

Ubiquitination of Pex5p only takes place at the peroxisomal membrane after receptor docking

Pex5p is predominantly cytosolic, with a portion also being localized at the peroxisomal membrane (for review see [4,40,41]). In order to analyse where in the cell the Pex5p ubiquitination takes place, we tested subcellular fractions of *pex1*Δ and *pex4*Δ and *pex1*Δ*pex4*Δ cells for the presence of Ub-Pex5p. The cells were lysed by treatment with glass beads, and the resulting crude extract was separated into a 100000 ***g*** supernatant (containing soluble proteins) and a membranous pellet fraction. These fractions were analysed for the presence of Pex5p and Pex13p, as well as cytosolic fructose-1,6-bisphosphatase (Fbp1p; [42]) as a control for proper separation. Pex13p, but not Fbp1p, did sediment, indicating the complete sedimentation of cytosol-free peroxisomal membranes (Figure 3A). In all strains (wild-type, *pex1*Δ and

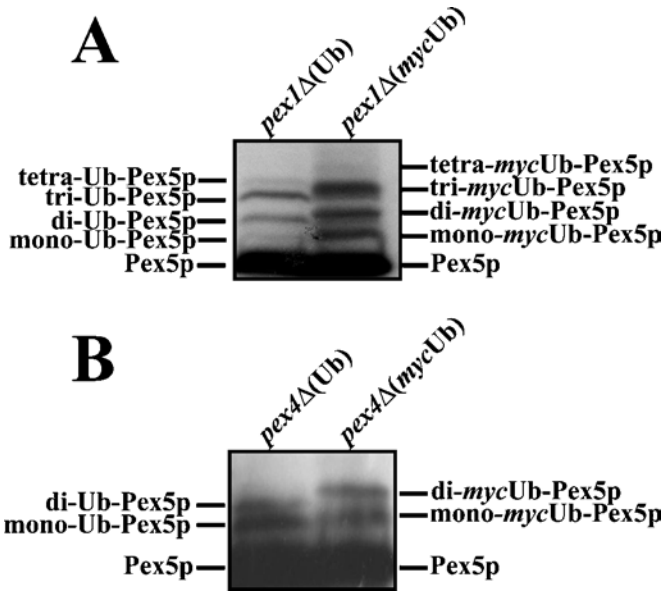


Figure 2 Ubiquitination of Pex5p

Cells of (**A**) *pex1*Δ or (**B**) *pex4*Δ bearing plasmids YEp96 and YEp105 [31] containing the genes coding for normal Ub or tagged Ub (*myc*Ub) respectively were grown in the presence of $CuSO_4$ to induce their expression from the *CUP1* promoter. Whole-cell trichloroacetic acid lysates were separated by SDS/PAGE, blotted on to nitrocellulose filters and labelled with anti-Pex5p antibodies. The shift of Pex5p forms in cells upon expression of *myc*Ub proves Pex5p to be ubiquitinated.

*pex4*Δ, as well as the *pex1*Δ*pex4*Δ double-mutant strain), Pex5p was localized to the 100000 ***g*** supernatant and pellet fraction. Ub-Pex5p found in the homogenate of these cells was totally recovered in the 100000 ***g*** sediment fraction. We conclude that Pex5p ubiquitination of Pex5p does not occur in the cytosol, but only takes place at the peroxisomal membrane.

Previously, it has been reported that the receptor docking complex consisting of Pex13p, Pex14p and Pex17p is associated in a Pex8p-dependent state with the three RING-finger proteins Pex2p, Pex10p and Pex12p, which also form a complex [43]. To investigate whether the ubiquitination of Pex5p already takes place after docking and whether it requires the presence of the RING-finger peroxins, we tested whether Ub-Pex5p is present in cells lacking Pex10p, an essential component of the RING-finger complex. Deletion of either one of the RING-finger proteins is known to dissociate the RING-finger complex, but docking of the receptors remains unaffected [44]. As shown in Figure 3(B), ubiquitinated Pex5p was not visible in whole-cell extracts of the *pex10*Δ strain (Figure 3B). The same was true for the combination of *pex4*Δ and *pex10*Δ. Taken together, our data demonstrate that Pex5p is ubiquitinated after receptor docking, and ubiquitination depends on the presence of Pex10p.

Ubiquitination of Pex5p in *pex1*Δ and *pex4*Δ cells depends on Ubc4p

The enzymic cascade for Ub conjugation is organized hierarchically. There is one Ub-activating enzyme (E1), a significant, but limited, number of Ub-conjugating enzymes (E2), and a much larger number of Ub ligases (E3). Each E3 recognizes a set of substrates that shares one or more ubiquitination signals, and co-operates with one or a few E2s (for review see [45]). In *S. cerevisiae*, there are 13 E2s known, one of which is Pex4p, also known as Ubc10p [24].

In order to analyse whether one of the known Ubc proteins is responsible for the Pex5p ubiquitination, combinations of *pex1*Δ or *pex4*Δ mutants with deletions of one of the *UBC* genes were constructed. Due to the fact that *ubc3*Δ and *ubc9*Δ exhibit a lethal

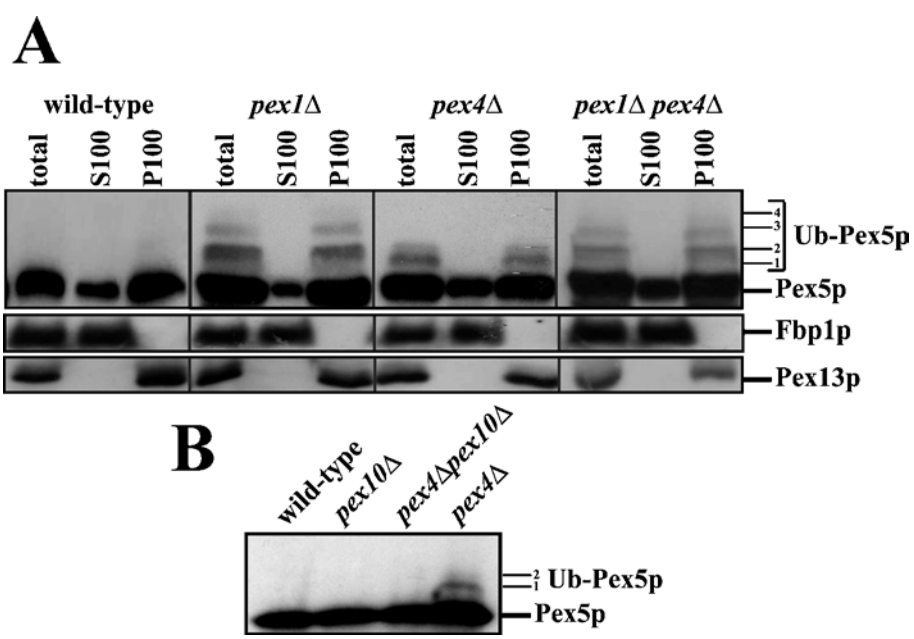


Figure 3 Pex5p ubiquitination takes place at the peroxisomal membrane after the docking step

(**A**) Homogenates of oleic-acid-induced cells derived from wild-type, *pex1*Δ, *pex4*Δ and *pex1*Δ*pex4*Δ were separated by differential centrifugation into a 100000 ***g*** supernatant (S100) and a pellet (P100) fraction. Equal portions of each fraction were analysed by immunoblotting. Pex13p, but no portion of the cytosolic fructose-1,6-bisphosphatase (Fbp1p), was found in the membrane sediment, indicative of proper separation. All Ub-Pex5p found in the homogenate (labelled 1–4) was recovered in the 100000 ***g*** pellet. (**B**) Whole-cell trichloroacetic acid lysates of wild-type, *pex10*Δ, *pex4*Δ and *pex4*Δ*pex10*Δ double-mutant strains were analysed for Pex5p ubiquitination by immunoblotting with anti-Pex5p antibodies. No Ub-Pex5p was visible in a *pex10*Δ or a *pex4*Δ*pex10*Δ strain, indicating that the presence of Pex10p is required for Pex5p ubiquitination.

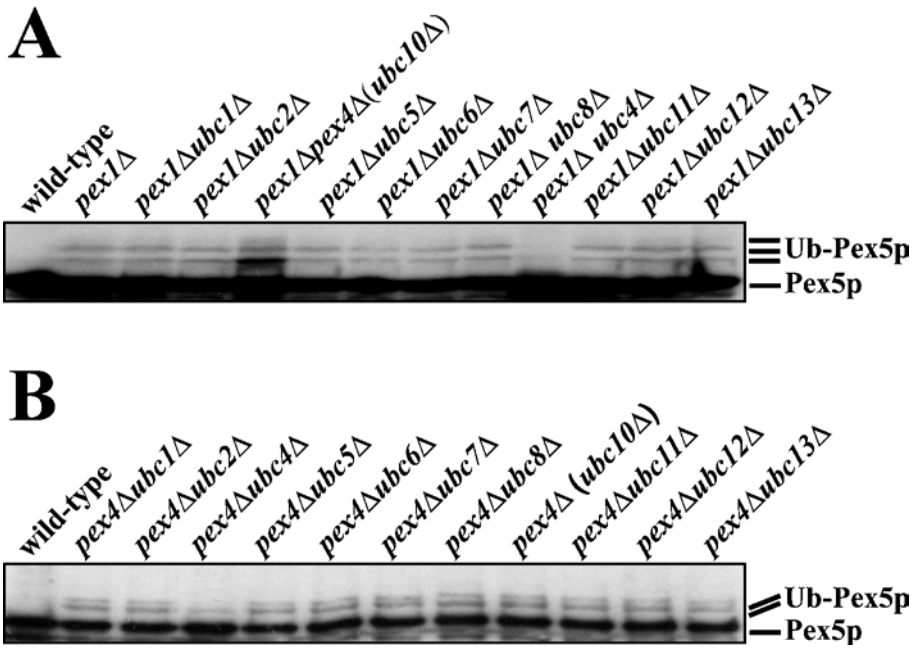


Figure 4 Pex5p ubiquitination depends on Ubc4p

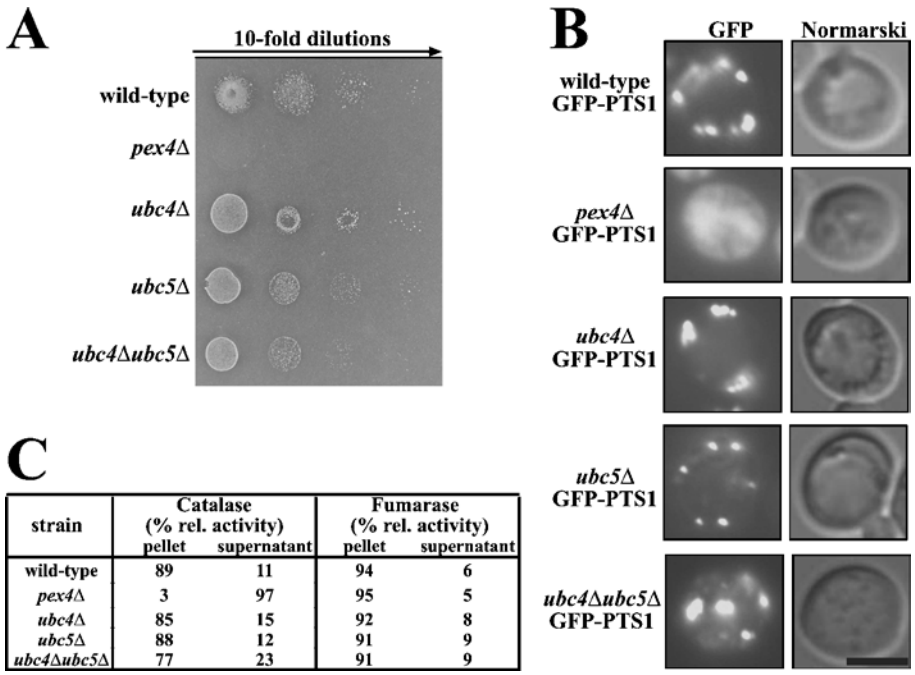
Analysis of (**A**) *pex1Δubc Δ* and (**B**) *pex4 Δubc Δ* double-mutants. Whole-cell lysates of indicated mutant strains were analysed for Pex5p ubiquitination by immunological detection with anti-Pex5p antibodies. Ub-Pex5p was significantly decreased in strains with deletions of *PEX1* or *PEX4* combined with deletion of *UBC4*.

phenotype [32,46,47], these combinations were omitted. Whole-cell lysates of the wild-type, *pex1Δ* and *pex1Δubc Δ* or *pex4Δ* and *pex4Δubc Δ* mutants were subjected to SDS/PAGE and immunologically probed for the presence of Pex5p and its ubiquitinated forms. The ubiquitinated Pex5p was clearly visible in samples derived from the *pex1Δ* or *pex4Δ* single mutant. With one exception, the combinations of *pex1Δ* and *pex4Δ* with the different *ubc Δ* did exhibit the same modification pattern as the corresponding single mutant strains of *pex1Δ* (Figure 4). Most interestingly, the modification pattern observed in the *pex1Δpex4Δ* strain appeared to be an assembly of the different patterns observed in the single mutants. However, deletion of either *pex1Δ* or *pex4Δ* combined with *ubc4Δ* reduced the Ub-Pex5p drastically. These data indicate that Ubc4p is required for Pex5p ubiquitination. One of the ubiquitinated forms of Pex5p was still visible in the *pex4Δubc4Δ* strain, although with a significantly reduced intensity. It has been reported that Ubc4p often exhibits overlapping function with Ubc5p [48]. However, deletion of both Ubcs together in a *pex4Δ* background did not result in a complete vanishing of the Ub-Pex5p (results not shown). This might be due to the fact that Ubc1p is also able to substitute for the lack of Ubc4p and Ubc5p [49].

Our data demonstrate that Ubc4p is involved in Pex5p ubiquitination. To determine whether deletion of *UBC4* also results in a loss of peroxisomal function, we analysed the growth behaviour of wild-type and different deletion strains on oleic acid as the sole carbon source. In contrast with wild-type cells, cells deficient in *PEX4* were unable to grow on oleic acid, as typical for *S. cerevisiae* mutant strains that are defective in peroxisome metabolism or biogenesis [28]. Strains *ubc4*Δ and *ubc5*Δ grew at the wild-type rate (Figure 5A). Notably, the *ubc4*Δ*ubc5*Δ strain demonstrated a slightly reduced growth on oleic acid, indicating that both proteins play a role in peroxisomal function (Figure 5A).

To elucidate further the basis of the reduced growth on oleic acid of the *ubc4*Δ*ubc5*Δ strain, we investigated the intracellular localization of peroxisomal matrix proteins. GFP–SKL (Ser-Lys-Leu) was used for analysing the PTS1- and therefore Pex5p-dependent import by fluorescence microscopy analysis. The protein was imported into peroxisomes of wild-type cells as demonstrated by the peroxisome-characteristic punctate staining (Figure 5B). As expected, GFP–SKL caused diffuse staining in the *pex4*Δ mutant, which is defective in PTS1- and PTS2-dependent import. Strains *ubc4*Δ and *ubc5*Δ were indistinguishable from wild-type cells in terms of their fluorescence pattern. However, when *ubc4*Δ and *ubc5*Δ were combined in a double-mutant strain, GFP–SKL caused a punctate staining like that in wild-type cells, but also a cytosolic fluorescence pattern (Figure 5B). These data suggest that a partial import defect is a consequence of combined deletion of *UBC4* and *UBC5*.

To quantify this import defect, we analysed the subcellular distribution of the peroxisomal matrix protein catalase (PTS1-containing peroxisomal matrix protein) by cell fractionation analysis of wild-type, *pex4*Δ, *ubc4*Δ, *ubc5*Δ and *ubc4*Δ*ubc5*Δ double-mutant strain. The different strains were grown on oleic acid and were subjected to subcellular fractionation to give rise to a 25000 ***g*** pellet enriched for peroxisomes and mitochondria, and a 25000 ***g*** supernatant enriched for cytosol. As expected, the majority of the peroxisomal matrix protein catalase as well as mitochondrial fumarase were detected in the 25000 ***g*** pellet of wild-type cells (Figure 5C). In contrast, in *pex4*Δ cells, catalase was preferentially localized to the 25000 ***g*** supernatant consistent with its mislocalization to the cytosol. Again, *ubc4*Δ and *ubc5*Δ cells were similar to wild-type strain in terms of the distribution of mitochondrial and peroxisomal marker proteins. However, deletion of both *UBC4* and *UBC5* led to a doubling in the amount of cytosolic catalase (Figure 5C). As in the *ubc4*Δ*ubc5*Δ strain, the distribution of mitochondrial fumarase between organellar pellet and cytosol was indistinguishable from samples derived from wild-type, indicating that the observation was not due to a higher breakage of organelles. Consequently, the sedimentation



C

strain	Catalase (% rel. activity) pellet	Catalase (% rel. activity) supernatant	Fumarase (% rel. activity) pellet	Fumarase (% rel. activity) supernatant
wild-type	89	11	94	6
pex4Δ	3	97	95	5
ubc4Δ	85	15	92	8
ubc5Δ	88	12	91	9
ubc4Δubc5Δ	77	23	91	9

Figure 5 Ubc4p and Ubc5p are required for proper protein import into peroxisomes

(**A**) Growth behaviour of *S. cerevisiae* strains on oleic acid as the sole carbon source. Serial dilutions of wild-type, *pex4*Δ, *ubc4*Δ, *ubc5*Δ and *ubc4*Δ*ubc5*Δ were spotted on to plates containing oleic acid, and incubated for 2–7 days at 30 °C. (**B**) Localization of a PTS1-reporter protein GFP–PTS1. Strains expressing GFP–PTS1 were examined for GFP fluorescence as indicated. Structural integrity of the cells is documented by bright-field microscopy. Scale bar, 5 μm. (**C**) Subcellular distribution of peroxisomal and mitochondrial marker enzymes in oleic-acid-induced strains. After centrifugation of cell-free homogenates at 25 000 ***g***, sediments and supernatants were assayed for peroxisomal catalase as well as for mitochondrial fumarase relative (rel.) activities.

data corroborate the fluorescence microscopic analysis supporting the notion that the lack of Ubc4p and Ubc5p results in a partial mislocalization of peroxisomal matrix proteins to the cytosol.

Polyubiquitination of Pex5p

Recognition of proteins destined for the Ub-mediated proteolysis pathway requires formation of a polyubiquitin chain. The Ub molecules are linked within this chain by isopeptide bonds connecting the C-terminal Gly^{76} of one Ub moiety to the ε-amino group of Lys^{48} of the adjacent Ub molecule [45]. Although Lys^{29} and Lys^{63} also have been found to function as sites for polyubiquitination [50], Lys^{48} is the primary site of this process. Ub with a substitution of Lys^{48} by an arginine (Ub-K48R) can still be conjugated to other proteins, but fails to function as an acceptor within the polyubiquitin chain [51]. Therefore Ub-K48R can serve as a probe for monitoring the presence of a Lys^{48}-linked polyubiquitin chain in a protein of interest. The appearance of several ubiquitinated Pex5p species suggested that these represent different states of ubiquitination. To investigate whether the observed modifications are generated by multiple mono-ubiquitination or by polyubiquitination, we compared the Pex5p pattern of *pex1*Δ and *pex4*Δ cells expressing either plasmid-encoded wild-type-Ub or Ub-K48R. The Pex5p pattern in both, *pex1*Δ and *pex4*Δ cells expressing wild-type Ub was indistinguishable from untransformed mutant cells. However, expression of Ub-K48R resulted in a significant decrease of the upper bands of Pex5p in *pex1*Δ cells (Figure 6A) and *pex4*Δ cells (Figure 6B), whereas the lower band was still visible or increased. The fact that the polyubiquitinated forms did not completely disappear upon expression of the Ub-R48K is explained by the fact that the endogenous wild-type Ub is still expressed, albeit much lesser than the mutant form. From these data, we conclude that linkage

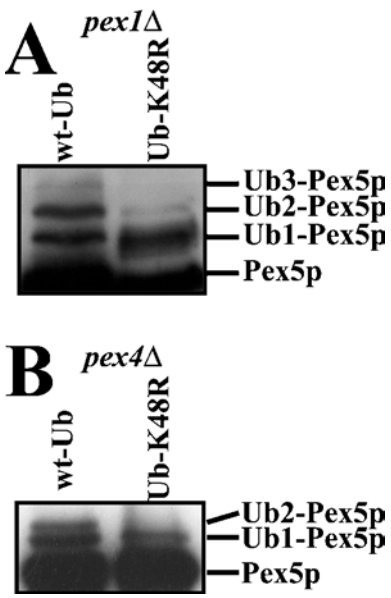


Figure 6 Polyubiquitination of Pex5p

(**A**) *pex1*Δ or (**B**) *pex4*Δ cells transformed with either a plasmid encoding wild-type Ub (wt-Ub) or mutant Ub (Ub-K48R) were grown in the presence of $CuSO_4$. Whole-cell lysates of the strains were analysed for the ubiquitination of Pex5p by immunological detection with anti-Pex5p antibodies. Expression of mutant Ub results in a disappearance of the upper Ub-Pex5p band(s), indicative of polyubiquitination of Pex5p.

of a polyubiquitin chain containing a Lys^{48} linkage is necessary to form the upper Pex5p-modification, which thus represents polyubiquitinated Pex5p, while the lower band most likely represents mono-ubiquitinated Pex5p.

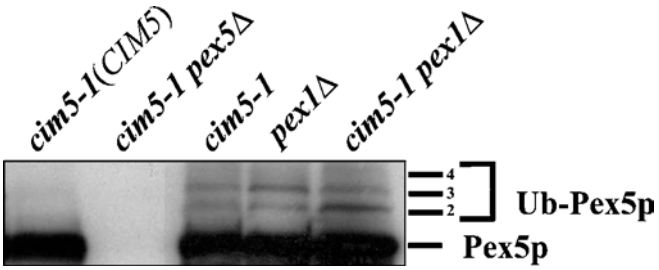


Figure 7 Ubiquitinated Pex5p accumulates in the proteasomal-mutant strain *cim5-1*

Strains were grown on oleic acid at the non-permissive temperature of 37 °C. Whole-cell trichloroacetic acid lysates were prepared and equal amounts thereof were separated by SDS/PAGE and blotted on to a nitrocellulose filter. The filter was probed for the presence of Pex5p and immunoreactive complexes were visualized with the ECL® system. Ubiquitinated forms of Pex5p are labelled 2–4.

Proteasomal degradation of ubiquitinated Pex5p

Lys[48]-dependent polyubiquitinated proteins are thought to be recognized and degraded by the proteasome, an essential high-molecular-mass protease present in the cytoplasm and the nucleus of all eukaryotic cells. Studies with proteasome inhibitors have shown that cellular proteins and their ubiquitinated species could be stabilized, when proteasome function, most likely that of the 20 S protease, which is the central protease in the Ub-dependent degradation process, is inhibited (reviewed in [52]). In order to elucidate whether Ub-Pex5p is also present in wild-type cells and thus reflects a physiologically relevant stage of the PTS1 receptor, we made use of a strain carrying a temperature-sensitive mutation in the *CIM5* (co-lethal in mitogenesis) gene. This gene encodes a regulatory subunit of the 26 S proteasome [53]. *Cim5-1* mutant cells arrest the cell cycle at non-permissive temperature and accumulate ubiquitinated proteins [54]. Whole-cell lysates of oleic-acid-induced *cim5-1* cells, as well as double mutants of *cim5-1* and either *pex1*Δ or *pex5*Δ, all grown at non-permissive temperature (37 °C), were analysed for the presence of modified Pex5p. No ubiquitinated Pex5p was labelled in samples derived from the *cim5-1* strain functionally complemented with a plasmid harbouring the *CIM5* gene, representing the wild-type situation (Figure 7). Accumulation of ubiquitinated Pex5p was observed in *cim5-1* and *cim5-1 pex1*Δ cells when grown at non-permissive temperature (Figure 7). When grown at the permissive temperature of 30 °C, Ub-Pex5 was not visible in *cim5-1* cells, but remained at the same level of intensity in *cim5-1/pex1*Δ cells (results not shown).

These data underline the involvement of the proteasome in the degradation of ubiquitinated Pex5p. Moreover, the data demonstrate that Pex5p-modification also takes place in strains which are not affected in peroxisomal biogenesis, and thus the Pex5p-ubiquitination is supposed to reflect a physiologically relevant stage in the Pex5p receptor cycle.

DISCUSSION

Different modifications of proteins involved in peroxisomal biogenesis have been reported previously. Pex14p has been shown to be phosphorylated in both *Hansenula polymorpha* and *P. pastoris* [55,56]. Farnesylation occurs to *S. cerevisiae* Pex19p [57], whereas mono- and di-ubiquitination of Pex18p is associated with a constitutive degradation of the protein in *S. cerevisiae* [26].

In the present paper, we demonstrate that the PTS1 receptor Pex5p is ubiquitinated at the peroxisomal membrane in cells lacking Pex1p, Pex6p, Pex15, Pex4p or Pex22p. Pex1p and Pex6p are AAAs that form a complex supposed to be anchored to the peroxisomal membrane via Pex15p [58]. In support of a close functional relationship of the three proteins, deficiency in one of the components leads to the same pattern of Pex5p modifications representing the di- and tri-ubiquitinated forms, as well as minor amount of mono- and tetra-ubiquitinated forms of Pex5p (Figure 1). This Pex5p pattern differed from the one obtained upon deletion of Pex4p or Pex22p, which mainly consists of the mono- and di-ubiquitinated forms. Pex4p and Pex22p also form a functional complex, with Pex4p being a putative Ub-conjugating protein, which is anchored to the peroxisomal membrane via Pex22p [38]. Given that the usual consequence of polyubiquitination is degradation, Pex5p should be less stable in the above mentioned mutants. We did not observe such an instability of Pex5p in *S. cerevisiae*; however, it has been reported to occur in exactly these mutants in human and *P. pastoris* cells [17,37,38]. Thus it also seems likely that in *S. cerevisiae*, the ubiquitination of Pex5p might prime the protein for degradation, but in contrast with human and *P. pastoris* cells, in the *S. cerevisiae pex* mutants, Pex5p degradation is not brought to completion.

We found that mono- and poly-ubiquitination of Pex5p depends on the presence of the Ub-conjugating enzyme Ubc4p, which is known to mediate selective proteasomal degradation of short-lived and abnormal proteins [48]. The involvement of the proteasome in the turnover of ubiquitinated Pex5p is indicated by the accumulation of ubiquitinated Pex5p in proteasomal mutants (Figure 7).

Our observation of the Pex5p-modification to be present in *pex4*Δ strain, but not in a *pex4*Δ*pex10*Δ double-mutant, indicates that Pex10p performs its function in the Pex5p-dependent protein import pathway before Pex4p. This is in line with the observation of Collins et al. [18] who used the instability of Pex5p to determine the epistatic relationships among several groups of *pex* mutants. Accordingly, the AAA- and the Pex4p-complexed proteins were to act late in the peroxisomal import pathway after docking and after the so far unspecified step in the import process performed by the RING-finger proteins. Deletion of either one of the RING-finger proteins is known to dissociate the RING-finger complex, but docking of Pex5p remains unaffected [43,44]. As ubiquitination of Pex5p was neither visible in whole-cell extracts of the *pex10*Δ mutant, nor in the extracts of the *pex4*Δ*pex10*Δ double-mutant, our data demonstrate that the ubiquitination of Pex5p takes place after receptor docking and that it depends on the presence of Pex10p. The requirement for Pex10p can be explained in two ways. As E3-ligases, RING finger proteins may play the primary role in determining substrate specificity for Ub conjugation [45]. Thus Pex5p could be a *bona fide* substrate for a putative E3 function of the RING finger peroxins. On the other hand, in cells lacking RING-finger peroxins, Pex5p remains associated with the docking complex [43]. The lack of Pex5p ubiquitination in these cells might therefore be due to the fact that Pex5p accumulates at an early stage of the import pathway. Thus Pex5p might not yet have reached a stage in the import cascade which requires or leads to dislocation and proteasomal disposal of the protein.

Pex5p is supposed to cycle between the cytosol and the peroxisomal compartment. According to the shuttle model of peroxisomal protein import, Pex5p binds its cargo in the cytosol, delivers it to the peroxisomal membrane and releases its cargo at a putative translocation complex [16,17]. According to the extended shuttle model, Pex5p enters the peroxisome in tandem with its cargo and releases the cargo in the peroxisomal lumen [59,60]. After cargo release, Pex5p is transported back to the cytosol in both scenarios. Gouveia et al. [61] reported that Pex5p exits the peroxisomal compartment in an ATP-dependent manner. We found

a significant amount of Pex5p to be membrane-associated in cells lacking components of the AAA complex (Figure 3). Thus the AAAs are good candidates for being responsible for the ATP-dependent recycling of Pex5p to the cytosol. Accordingly, components of the AAA complex as well as the Pex4p–Pex22p complex have been reported to be required for the late steps in the import process [18].

What is the functional role of the observed ubiquitination of Pex5p? First, ubiquitination of Pex5p might represent an intrinsic step in the peroxisomal protein import pathway. Although this might well be true and Pex5p might be the long sought *bona fide* substrate for Pex4p, our data do not provide conclusive evidence of this. At first sight, the major appearance of mono- and di-ubiquitinated forms of Pex5p in cells lacking Pex4p or Pex22p, but the nearly complete lack of the mono-ubiquitinated form and the appearance of tri- and tetra-ubiquitinated forms in the cells lacking components of the AAA complex, might suggest that Pex4p is specifically required for the polyubiquitination of Pex5p. In this respect, it is interesting to note that Pex4p has recently been reported to be required for di-ubiquitination of Pex18p [26]. However, the Pex5p-ubiquitination pattern in a *pex1Δpex4Δ* double-mutant (Figure 4) resembles a mixture of both pattern types. This, however, might be explained by the assumption that deficiency in the different components leads to the ubiquitination of different sites within Pex5p. In this case, Pex4p could be responsible for the polyubiquitination of Pex5p at one site in cells lacking components of the AAA complex, while in the absence of Pex4p, the protein is polyubiquitinated at another site. It will be of great interest to follow up this possibility in terms of the function of Pex4p in peroxisomal protein import. However, our observation also demonstrates that polyubiquitination of Pex5p can, in principle, also take place in the absence of Pex4p. Consequently, we have to consider that the observed Pex5p ubiquitination in mutants affected late in the import pathway is due to the fact that Pex5p might get stuck in the import pathway, leading to an aberrant accumulation of membrane-associated Pex5p. This would also be in line with the observed involvement of Ubc4p, which is known to be responsible for the proteasomal disposal of abnormal proteins. This, however, leads to the question of why ubiquitinated Pex5p is only observed in import mutants affected in late steps of the import pathway. In this respect, it is interesting to note that Gouveia et al. [61] distinguished three different stages of Pex5p association with the peroxisomal membrane, with stage 3 being predominantly detected under ATP-limiting conditions. Under these conditions, Pex5p has been reported to be a target for an ATP-utilizing component mediating its release from the peroxisomal compartment. Although the identity of this ATPase is not known, the AAA-peroxins are good candidates to perform this function. Based on these observations, we propose that the lack of the late components of the import cascade leads to a membrane accumulation of Pex5p at a stage which sets the signal for its ubiquitination and intended proteasomal degradation. Ubc4p has also been implicated in ubiquitination and constitutive degradation of Pex18p, a PTS2-specific peroxin, the function of which in peroxisome biogenesis is accompanied by its rapid turnover [26]. The mechanistic interplay of Pex18p ubiquitination and its function in protein import into peroxisomes, however, has not yet been elucidated. The observation that both Pex5p and Pex18p, two peroxins which are supposed to cycle between the cytosol and the peroxisomal membrane, are ubiquitinated gives rise to the idea that their turnover might be of importance for their functional role in peroxisomal protein import. Moreover, the Ub-Pex5p accumulation in proteasomal mutants demonstrates that the Pex5p-modification also takes place in strains that are not affected in peroxisomal biogenesis. Consequently, we propose that the ubiquitination of Pex5p represents a genuine stage in the Pex5p receptor cycle. The data presented here are clear in that Pex5p is modified by ubiquitination at the peroxisomal membrane when peroxisomal import is blocked at late stages or upon inhibition of proteasomal degradation; however, additional work will be needed to clarify the physiological relevance of this modification.

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Functional role of the AAA peroxins

in dislocation of the cycling PTS1-receptor back to the cytosol

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LETTERS

Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol

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Peroxisomal import receptors bind their cargo proteins in the cytosol and target them to docking and translocation machinery at the peroxisomal membrane (reviewed in ref. 1). The receptors release the cargo proteins into the peroxisomal lumen and, according to the model of cycling receptors, they are supposed to shuttle back to the cytosol. This shuttling of the receptors has been assigned to peroxins including the AAA peroxins Pex1p and Pex6p, as well as the ubiquitin-conjugating enzyme Pex4p (reviewed in ref. 2). One possible target for Pex4p is the PTS1 receptor Pex5p, which has recently been shown to be ubiquitinated[3–5]. Pex1p and Pex6p are both cytosolic and membrane-associated AAA ATPases of the peroxisomal protein import machinery, the exact function of which is still unknown. Here we demonstrate that the AAA peroxins mediate the ATP-dependent dislocation of the peroxisomal targeting signal-1 (PTS1) receptor from the peroxisomal membrane to the cytosol.

The peroxisomal import of folded, even oligomeric proteins is an ATP-dependent process, which so far is only poorly understood. Conceptually the process is divided into four steps: (1) cargo recognition; (2) docking of the cargo-loaded receptors to the peroxisomal membrane; (3) cargo release and translocation; and (4) receptor recycling. Recent data indicate that the ATP is not required for the first three steps, but is needed for returning the import receptor to the cytosol[6–8]. Because of their ATPase activity, we suspected the AAA peroxins Pex1p and Pex6p to catalyse this ATP-dependent step in the protein import pathway into peroxisomes. This would also be in agreement with the previously described epistasis analysis[9], which placed the function of the AAA peroxins at the end of the receptor cycle. To analyse the function of the AAA peroxins in this context, oleic-acid-induced cells of *Saccharomyces cerevisiae* wild type, *pex1Δ*, *pex6Δ*, *pex15Δ*, *pex13Δ14Δ* and *pex8Δ* were disrupted with glass beads and the corresponding homogenates were separated into a 100,000*g* supernatant and a membranous pellet fraction. As a control for proper separation, these fractions were analysed for the presence of the integral peroxisomal membrane protein Pex11p[10,11] as well as cytosolic fructose-1,6-bisphosphatase (Fbp1p[12]). Pex11p but not Fbp1p pelleted, indicating the complete sedimentation of cytosol-free peroxisomal membranes (Fig. 1a). Pex5p was detected in both cytosolic and membrane fractions, but the distribution differed between the strains. In the wild-type and *pex8Δ* strain, Pex5p was equally distributed between the cytosolic and membrane fraction. Deletion of two components of the peroxisomal docking complex, Pex13p and Pex14p (reviewed in refs 13, 14), resulted in an almost complete cytosolic Pex5p localization, therefore underlining the function of these proteins in receptor docking. However, Pex5p was predominantly membrane-associated in strains deleted in Pex1p, Pex6p or Pex15p, the peroxisomal membrane anchor of Pex6p[15], an effect that was not due to a different Pex5p level of the different strains (see Supplementary Information, Fig. S1a). We also demonstrate that Pex1p, Pex6p and Pex15p together with Pex5p form a membrane-associated complex in wild-type cells (Fig. 1b). This association of Pex5p with the membrane-bound AAA complex, together with the accumulation of Pex5p at the peroxisomal membrane of AAA mutant strains, supports the notion that Pex1p and Pex6p function late in the import pathway, most probably in the release of receptors into the cytosol.

Previously, we and others have demonstrated the ubiquitination of the PTS1 receptor Pex5p in cells that lack components of the peroxisomal AAA complex[3,4]. Pex5p is ubiquitinated after the docking event and Ub-Pex5p accumulates at the peroxisomal membrane. Ubiquitinated Pex5p also accumulates in proteasomal mutants, thereby underlining the involvement of the proteasome in the degradation of ubiquitinated Pex5p. Cells deficient in either one of the components of the AAA complexes, Pex1p, Pex6p or Pex15p, exhibit the same ubiquitination pattern as a *cim5-1* strain, a temperature-sensitive strain that is defective in proteasomal degradation[16]. However, in cells defective in either component of the AAA complex, Ub-Pex5p is exclusively membrane associated, whereas a significant portion of Ub-Pex5p accumulates in the cytosol in the proteasome mutant strain (Fig. 1c). The combination of *cim5-1* with *PEX1* deletion exhibits the same localization of Ub-Pex5p as a *pex1Δ* strain. These findings demonstrate that Pex1p is not required for Pex5p ubiquitination but for Ub-Pex5p release from the peroxisomal membrane.

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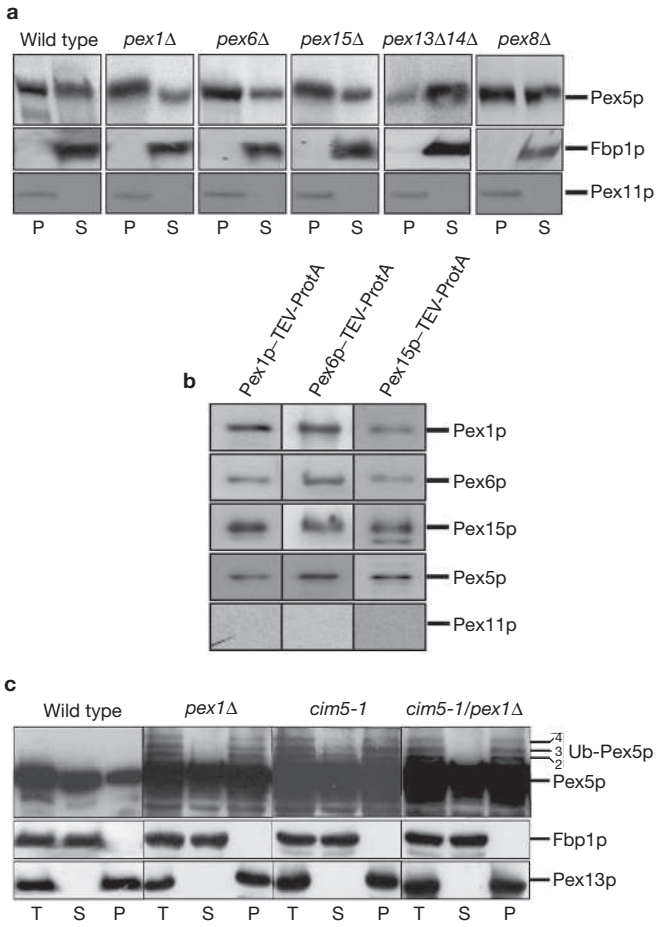

Figure 1 Pex5p forms a complex with the AAA group peroxins and accumulates at the peroxisomal membrane in their absence. Homogenates (T) derived from strains as indicated were separated by differential centrifugation into supernatant (S) and pellet (P) fractions. (**a**) Equal portions of each fraction were analysed by immunoblotting for the presence of Pex5p and marker proteins for membrane (Pex11p) and cytosol (Fbp1p). (**b**) Protein complexes were isolated from digitonin-solubilized membrane fractions of strains expressing Pex1p, Pex6p or Pex15p fused to TEV-ProtA. (**c**) Fractions of indicated strains were probed with Pex5p antibodies, and ubiquitinated Pex5p (Ub-Pex5p) forms were visualized and numbered 2–4, corresponding to their ubiquitination state.

To confirm the Pex1p/Pex6p-dependent recycling of Pex5p from the peroxisomal compartment back to the cytosol, an *in vitro* export assay was established. Making use of the fact that Pex5p ubiquitination exclusively takes place at the peroxisomal membrane at a later step in the import cascade[3], we were able to define this modification as a marker for the Pex5p population that has not yet left the peroxisomal membrane. The basis for the export assays were membranes prepared from *PEX1*/*PEX6*-null cells. These membranes, which accumulate Ub-Pex5p to the same degree as in *pex1Δ*, *pex6Δ*, or *pex15Δ* strains[3], were resuspended in either buffer or cytosol of wild-type cells. The reaction mixture was incubated in the presence or absence of an ATP-regenerating system for 30 min at 37 °C. Subsequently, the mixture was separated by centrifugation at 100,000*g* into a membrane pellet fraction and a cytosolic supernatant.

Incubation of the membranes with cytosol from a *pex1Δ6Δ* strain had no effect on the distribution of Ub-Pex5p, whereas incubation with wild-type cytosol did result in the disappearance of the ubiquitinated Pex5p species from the organellar fraction (Fig. 2a). However, Ub-Pex5p did not appear in the supernatant, which led to the assumption that Ub-Pex5p is removed from the membrane in an AAA-peroxin-dependent manner and is degraded in the cytosol by the 26S proteasome. In support of this hypothesis, addition of the proteasome inhibitor MG 132 to the experimental set-up did result in the disappearance of membrane-bound Ub-Pex5p but the protein was now clearly detected in the cytosolic fraction (Fig. 2a). This result confirms the functional role of AAA peroxins in Ub-Pex5p dislocation, but it also shows that the 26S proteasome itself is not actively involved in the extraction of Ub-Pex5p from the peroxisomal membrane, which has been suggested to be the case for some endoplasmic-reticulum-associated degradation (ERAD) substrates at the ER membrane[17].

To investigate whether the previously reported ATP dependence of the Pex5p export step[6,7] is also maintained in our *in vitro* assay, the same experiment was repeated in the absence of an ATP-regenerating system. When the ATP-regenerating system was omitted, the Ub-Pex5p remained completely in the organellar pellet fraction (Fig. 2a). These data clearly indicate that the AAA peroxins are required for the dislocation of Ub-Pex5p from the peroxisomal membrane to the cytosol in an ATP-dependent manner.

Next, we tested for the membrane localization of Ub-Pex5p and found the protein to be completely carbonate resistant in the *pex1Δ6Δ* mutant strain (Fig. 2b). Thus, Ub-Pex5p behaves as an integral membrane protein, which in the *in vitro* export assay is released in an AAA-dependent manner.

These data so far indicated that the AAA peroxins would be required to dislocate the polyubiquitinated integral Pex5p, which would then be directed to proteasomal disposal. Because the ubiquitinated forms only represent a very minor fraction of the membrane-bound Pex5p, we suspected that this would not be the main function of the AAA peroxins and so tested whether these proteins would also affect the localization of the membranous non-ubiquitinated Pex5p pool. For this we performed the export assay with Pex5p-deficient cytosol from a *pex5Δ* mutant and monitored the non-ubiquitinated forms with lower exposures. Under these conditions, the Pex5p that showed up in the soluble fraction could only be derived from the added membranes.

When the *pex1Δ6Δ* membranes were incubated with only buffer and ATP, Pex5p almost completely remained in the membrane pellet (Fig. 2c). When the membranes were incubated with *pex5Δ* cytosol containing the Pex1p/Pex6p complex in the presence of an ATP-regeneration system, more than half of the Pex5p was visible in the supernatant fraction. In the absence of the ATP-regenerating system, however, Pex5p remained completely in the organellar pellet fraction. Thus, these data suggest that polyubiquitination seems not to be a precondition for the removal of the protein from the peroxisomal membrane. In this respect, it is tempting to speculate that the recently observed mono-ubiquitination[5] might have a role as a signal for the export of Pex5p, but this remains to be investigated. Our data clearly show that Pex5p is dislocated from the peroxisomal membrane in the presence of cytosol in an AAA-peroxin- and ATP-dependent manner. Moreover, after performing the export assay, tests for the membrane localization of the remaining Pex5p revealed that most of the carbonate-resistant Pex5p had been removed (see Supplementary Information, Fig. S1B). This result indicates that membrane-integrated Pex5p is a major target for Pex1p/Pex6p-dependent export.

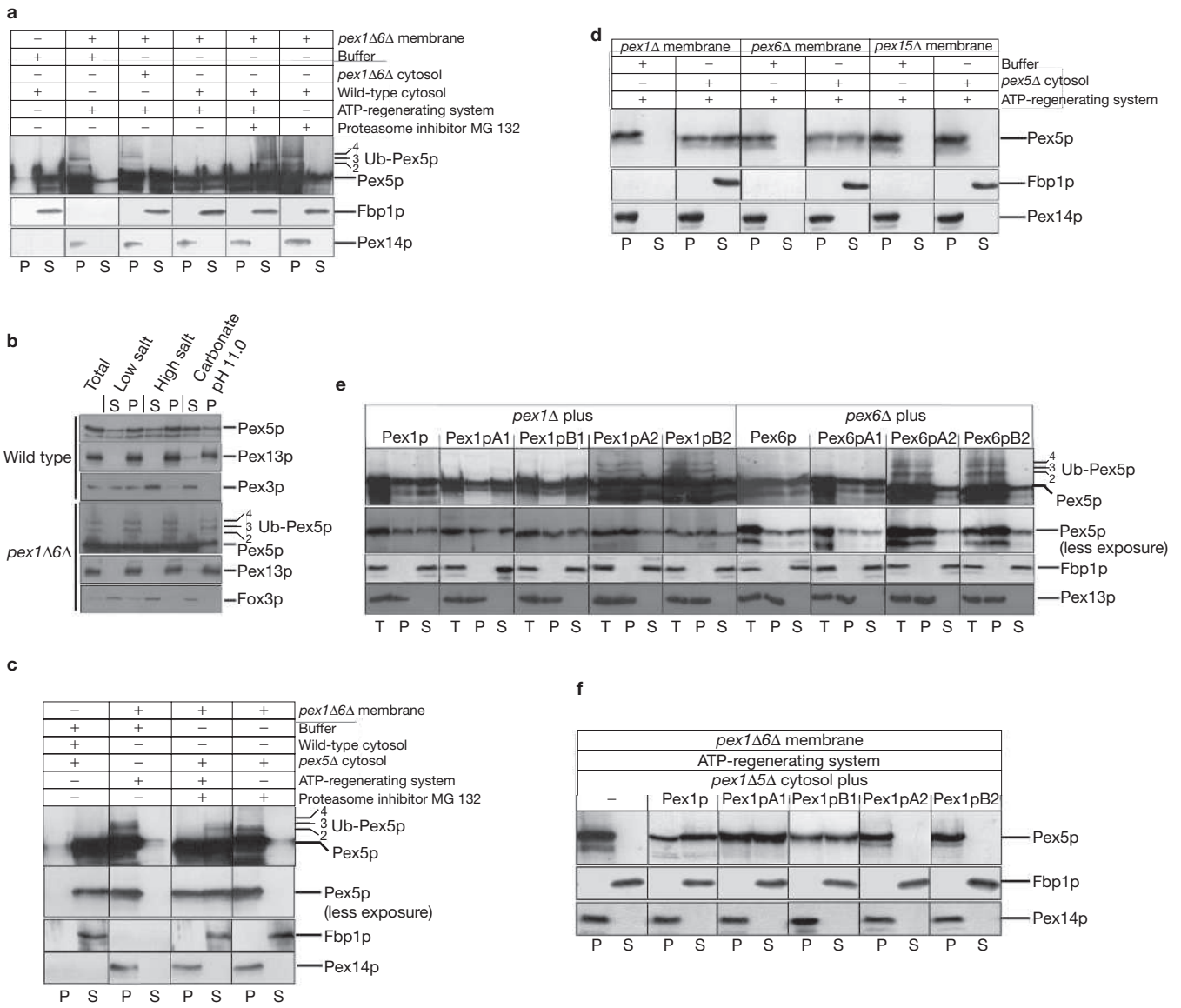

Figure 2 *In vitro* export assays and localization studies of Pex5p. (**a**, **c**) Export assays were performed with membranes from *pex1Δ6Δ* cells and cytosol derived from wild-type (**a**) or *pex5Δ* cells (**c**). (**b**) Sedimented peroxisomes were extracted with different buffers. (**d**) Export assays were performed with membranes (as indicated) and cytosol derived from *pex5Δ* cells. (**e**) Cytosolic supernatant (S) and organellar pellet (P) fractions of strains containing AAA peroxins with indicated mutations — in either the ATP-binding (A) or hydrolysis domain (B) of the first non-conserved (1) or second conserved (2) AAA cassette — were analysed for the presence of Pex5p and for marker proteins for membrane (Pex13p) and cytosol (Fbp1p). (**f**) Export assays were performed with membranes from *pex1Δ6Δ* and cytosol derived from *pex1Δ5Δ* cells containing either wild-type or mutated Pex1p.

We also tested whether deficiency in single components of the peroxisomal AAA complex would affect the export capability of peroxisomal membrane. In the presence of *pex5Δ* cytosol, Pex5p was easily released from membranes that were prepared from either *pex1Δ* or *pex6Δ* single mutants (Fig. 2d). Interestingly, removal of Pex5p was impossible with membranes that lacked Pex15p, in line with the functional role of Pex15p as a peroxisomal membrane anchor for the AAA complex (Fig. 2d).

Pex1p and Pex6p both contain two AAA domains with different degrees of conservation. However, because overexpression of either of the AAA peroxins cannot functionally substitute for the absence of the binding partner, Pex1p and Pex6p do not have redundant functions (see Supplementary Information, Fig. S1C). To gain more insight into the functional role of the AAA cassettes, we studied variants thereof that were mutated in either the ATP-binding or hydrolysis domain of the first non-conserved or second conserved AAA cassette. *In vivo* studies revealed that cells harbouring AAA peroxins with mutations in the first cassettes exhibit wild-type localization of Pex5p, suggesting that ATP binding at the non-conserved domains is dispensable for the dislocase function of the AAA peroxins (Fig. 2e). In contrast, when the conserved cassettes were mutated, Pex5p was predominantly membrane associated and Ub-Pex5p appeared and accumulated at the peroxisomal membrane, as in the *pex1Δ* and *pex6Δ* mutants. These data were corroborated by *in vitro* studies demonstrating that the export of Pex5p was impaired when Pex1p harboured the mutations within the second cassette (Fig. 2f). These observations indicate that ATP binding and hydrolysis by the second conserved AAA domain of the AAA peroxins is indispensable for the functional role of the proteins in Pex5p dislocation.

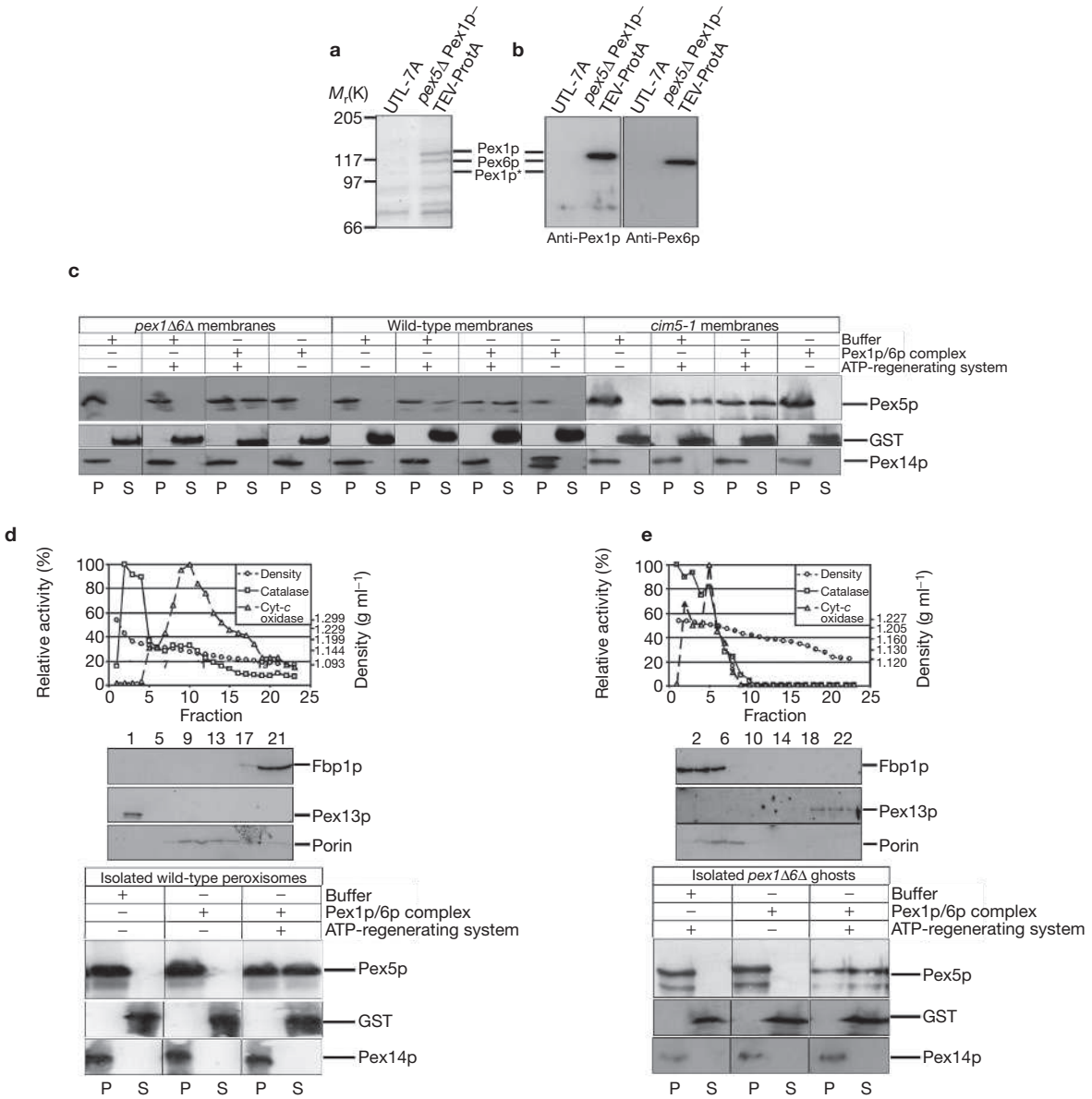

Figure 3 The AAA peroxins dislocate Pex5p from the peroxisomal membrane to the cytosol. (**a**–**e**) The AAA complex was purified from a cytosolic fraction of *pex5Δ* cells using the TEV-ProtA tag. Proteins were visualized with colloidal Coomassie (**a**) or western blot analysis (**b**). The asterisk marks a putative Pex1p degradation product. The purified AAA complex was used for export assays, performed with wild-type, *pex1Δ6Δ* or *cim5-1* membranes (**c**), wild-type peroxisomes separated by density gradient centrifugation (**d**), or *pex1Δ6Δ* remnants (ghosts) isolated from a flotation gradient (**e**). Fbp1p and porin served as marker for the cytosol and mitochondria, respectively. Pex14p served as marker for the membrane fraction, and GST was added to the export assays as an internal control for the soluble fraction. P, pellet; S, supernatant.

Next we analysed whether the Pex1p/Pex6p complex alone can substitute for the requirement of cytosol in Pex5p dislocation from the peroxisomal membrane. Cytosolic Pex1p fused to TEV-ProtA (Pex1p–TEV-ProtA) and associated proteins were isolated from *pex5Δ* cells via immunopurification. The isolated complex consisted of two dominant bands with an approximate relative molecular mass of 117,000 (M_r 117K) that appeared upon separation on SDS gels and Coomassie staining (Fig. 3a). The bands were immunologically identified as Pex1p and Pex6p (Fig. 3b), in line with the finding that both proteins have been shown to interact and are thought to form a heteromeric complex[18,19].

Remarkably, addition of the isolated Pex1p/Pex6p complex to wild-type, *pex1Δ6Δ* or *cim5-1* membranes resulted in the release of the main portion of Pex5p, including the polyubiquitinated forms (data not shown) from the membrane that appeared as dislocated Pex5p in the supernatant fraction (Fig. 3c).

In the presence of an ATP-regeneration system, a small portion of Pex5p was already removed from wild-type and *cim5-1* membranes. This can easily be explained by the fact that these membranes still contain the endogenous AAA peroxins. Therefore, the presence of ATP is sufficient to remove some Pex5p from these membranes but not from membranes derived from *pex1Δ6Δ* cells that lack the AAA peroxins. In the absence of the ATP-regenerating system, no Pex5p was removed even in the presence of the endogenous AAA proteins (Fig. 3c). The same was true for isolated peroxisomes or peroxisomal membranes derived from a wild-type or *pex1Δ6Δ* mutant strain, respectively (Fig. 3d, e). These data clearly demonstrate that the heteromeric complex of the AAA

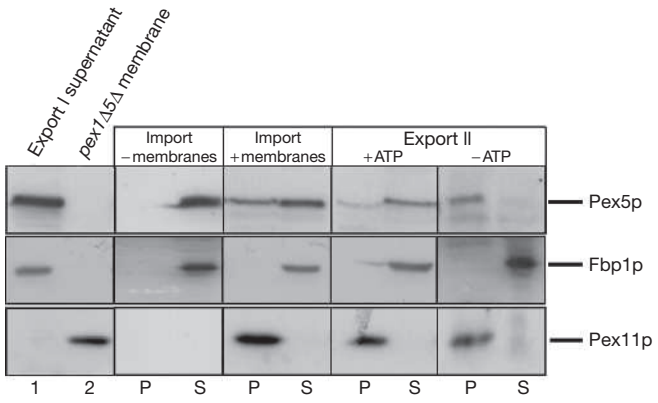

Figure 4 Cycling of the PTS1 receptor Pex5p between the peroxisomal membrane and the cytosol. For peroxisomal binding, Pex5p released from *pex1Δ6Δ* membranes (lane 1) was incubated with whole-cell membranes that were isolated from *pex1Δ5Δ* cells (lane 2) in the absence of an ATP-regenerating system. Subsequently, bound Pex5p was released from the membranes with *pex5Δ* cytosol in the presence but not in the absence of an ATP-regenerating system. Equal portions of the pellet (P) and supernatant (S) fractions were analysed by western blot for the presence of Pex5p and the indicated markers for cytosol (Fbp1p) and membrane (Pex11p).

peroxins functions as dislocase, which removes peroxisomal Pex5p from the peroxisomal membrane in an ATP-dependent manner.

The function of the AAA peroxins as Pex5p dislocase is consistent with an essential role of the proteins in the peroxisomal import pathway where they are required for the late steps in the Pex5p receptor cycle. According to our data, the AAA peroxins are responsible for ATP-dependent recycling of membrane-bound Pex5p back to the cytosol, where it is then available for another round of protein import into the organelle. An *in vitro* reconstitution of the complete Pex5p cycle comprising the ATP-dependent Pex5p release from the peroxisomal membrane, the subsequent ATP-independent membrane association of the released Pex5p, and finally the AAA- and ATP-dependent release of the newly bound Pex5p is shown in Fig. 4. This demonstrates that Pex5p is capable of performing multiple rounds of cycling between the peroxisomal membrane and the cytosol.

Our data clearly show that AAA peroxins perform the dislocation of Pex5p from the peroxisomal membrane. It is tempting to speculate that the ATP dependence of the dislocation step might be responsible for the energy requirement of the overall process of peroxisomal protein import. It will be challenging to elucidate how the ATP-dependent receptor dislocation is mechanistically linked to the translocation of folded proteins across the peroxisomal membrane.

METHODS

Strains, plasmids and culture conditions. The *S. cerevisiae* strain UTL-7A (MATa, ura3-52, trp1, leu2-3/112)[20] was used as an isogenic wild-type strain for the generation of *PEX1::PEX1*-TEV-ProtA (this study), *PEX6::PEX6*-TEV-ProtA (this study), *PEX15::PEX15*-TEV-ProtA (this study), *pex1Δ* (ref. 3), *pex1Δ/5Δ* (this study), *pex1Δ/6Δ* (this study), *pex5Δ* (ref. 21), *pex5Δ PEX1::PEX1*-TEV-ProtA (this study), *pex6Δ* (ref. 15), *pex8Δ* (ref. 22), *pex13Δ/14Δ* (this study) and *pex15Δ* (ref. 21). *cim5-1* (*cim5-1, ura3-52, leu2Δ 1, his3Δ200*)[16] served as an isogenic strain for *cim5-1/pex1Δ* (ref. 3) generation. Deletion strains were generated by the 'short flanking homology' method as previously described[23]. Strains in which the genomic copies of genes express proteins fused to TEV-ProtA were produced according to ref. 24. Cloning strategies and sequences of primers are available upon request. Yeast media have been described previously[20].

Plasmids expressing Pex1p, Pex1pA1^{K467E}, Pex1pB1^{D525Q}, Pex1pA2^{K744E}, Pex1pB2^{D797Q}, Pex6p (from a high-copy plasmid)[19], Pex6p, Pex6pA1^{K489A}, Pex6pA2^{K778A}, Pex6pB2^{D831Q} (ref. 15), or Pex1p (from a high-copy plasmid)[25] were used.

Immunopurification of native complexes using IgG-Sepharose. The method for the purification of the cytosolic Pex1p/Pex6p complex has been described previously[26]. In brief, 3 g of oleate-induced cells were lysed using glass beads and lysis buffer A containing protease inhibitors[26]. After sedimentation of the cell debris, the supernatant was centrifuged at 100,000*g* at 4 °C for 1 h (Sorvall AH-650; Sorvall, Bad Horneburg, Germany). For affinity chromatography, the generated supernatant was incubated overnight at 4 °C with 140 µl *hs*IgG-Sepharose slurry (Pharmacia, Uppsala, Sweden). The bound protein complex was washed and eluted by using 70 U TEV protease (Invitrogen, Carlsbad, CA), and 21 µl buffer followed by incubation at 16 °C for 2 h. TEV protease eluates were collected by centrifugation and stored at −80 °C until use.

***In vitro* import and export assay.** For export reaction, cytosol and membrane fractions were prepared according to ref. 3. Pex5p-containing membranes were derived from 300 mg cells and solubilized depending on the experimental condition in 1 ml of buffer, cytosol from an equal amount of cells from different yeast strains or with a comparable concentration of purified cytosolic AAA complex. Where indicated, MG 132 (Sigma, St Louis, MO) was added at a final concentration of 100 µM. ATP dependence was assayed in the presence of an ATP-regenerating system (10 mM creatin phosphate, 5 mM $MgCl_2$, 5 mM ATP, 5 U ml^{-1} creatin phosphokinase). Samples were incubated for 25–30 min at 37 °C and shaken gently. In a second step, cytosolic and membrane fractions were separated via centrifugation (100,000*g* for 30 min; Sorvall AH-650). When purified, peroxisomal membranes were used for export reaction, gradient fractions 2–3 (wild type) or 19–22 (*pex1Δ6Δ*) were pooled, and diluted at 1:5 with export buffer.

For import reactions, whole-cell membranes derived from 300 mg cells were diluted in buffer without ATP-regenerating system and incubated with the cytosol of an equal amount of cells at 26 °C for 25 min by gently shaking. Subsequent samples were fractionated into membrane and soluble supernatant fractions. Proteins were precipitated with trichloroacetic acid and samples were resolved in SDS–PAGE buffer.

Isolation and extraction of peroxisomes and peroxisomal remnants. Preparation of yeast spheroplasts, cell homogenization and determination of the suborganellar localization of proteins were performed according to ref. 10. For density gradient centrifugation of wild-type peroxisomes, a postnuclear supernatant was loaded on preformed 2.25–22.5% (w/v) Optiprep (iodixanol) gradients, as described in ref. 27, containing protease inhibitor mix[26] instead of EDTA. Peroxisomal remnants derived from a *pex1Δ6Δ* strain were floated up according to ref. 21. One-third of each gradient fraction was applied for enzyme and refractive index measurements followed by conversion to the respective density as well as western blot analysis, as described by ref. 20. The enzyme activity of catalase (EC 1.11.1.6) and cytochrome *c* oxidase (EC 1.9.3.1) were measured according to ref. 27.

Miscellaneous. Recombinant glutathione *S*-transferase (GST) was expressed as soluble protein from pGEX-4T-1 in *Escherichia coli* Bl21(DE3) and purified according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). TCA lysates of cellular fractions were prepared as previously described[3]. Immunoreactive complexes were visualized using anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase in combination with the ECL system from Amersham Pharmacia Biotech (Uppsala, Sweden). Polyclonal rabbit antibodies were against Pex1p, Pex6p[15], Pex5p, Pex11p, Pex13p[21], Pex14p[28], Pex15p[15], Fox3p[29] and fructose-1,6-bisphosphatase[30]. Monoclonal mouse antibodies were against actin (Sigma), porin[26] and GST (Sigma, Munich, Germany).

BIND identifiers. Three BIND identifiers (www.bind.ca) are associated with this manuscript: 300956, 300961 and 300962.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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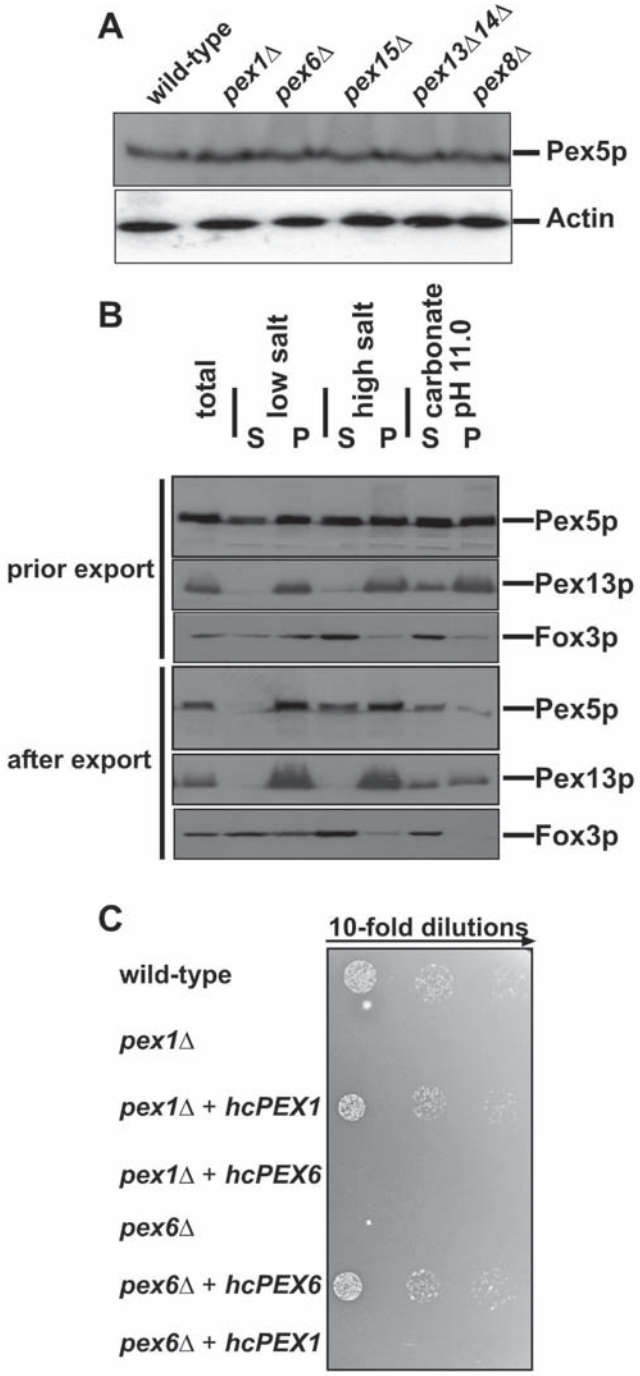

Figure S1 (A) Equal portions of homogenates derived from strains as indicated were analyzed for the presence of Pex5p and actin. The Pex5p-level was the same in all the strains. **(B)** Sedimented membranes were extracted either prior or after performed export assay with different buffer. Almost all carbonate resistant Pex5p was absent after export. **(C)** Cross complementation of *pex1Δ* and *pex6Δ* mutant. Pex1p and Pex6p were expressed from high copy plasmids and strong promoters and growth of cells was tested on oleic acid plates.

2.3

Membrane association of the cycling peroxisome import receptor Pex5p.

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Membrane Association of the Cycling Peroxisome Import Receptor Pex5p*

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Peroxisomal proteins carrying a peroxisome targeting signal type 1 (PTS1) are recognized in the cytosol by the cycling import receptor Pex5p. The receptor-cargo complex docks at the peroxisomal membrane where it associates with multimeric protein complexes, referred to as the docking and RING finger complexes. Here we have identified regions within the *Saccharomyces cerevisiae* Pex5p sequence that interconnect the receptor-cargo complex with the docking complex. Site-directed mutagenesis of the conserved tryptophan residue within a reverse W*XXX*F motif abolished two-hybrid binding with the N-terminal half of Pex14p. In combination with an additional mutation introduced into the Pex13p-binding site, we generated a Pex5p mutant defective in a stable association not only with the docking complex but also with the RING finger peroxins at the membrane. Surprisingly, PTS1 proteins are still imported into peroxisomes in these mutant cells. Because these mutations had no significant effect on the membrane binding properties of Pex5p, we examined yeast and human Pex5p for intrinsic lipid binding activity. *In vitro* analyses demonstrated that both proteins have the potential to insert spontaneously into phospholipid membranes. Altogether, these data strongly suggest that a translocation-competent state of the PTS1 receptor enters the membrane via protein-lipid interactions before it tightly associates with other peroxins.

Peroxisomes post-translationally import folded and oligomeric proteins of very different sizes from the cytosol across the single membrane into their matrix (1–4). This is in contrast to most other translocation systems that transport unfolded polypeptide chains (5). Although the identities of many proteins, collectively called peroxins, that are required for this process are known, the mechanism of protein translocation across the peroxisomal membrane is poorly understood.

Current evidence favors a cycling receptor model for matrix protein import (6–8). Two soluble import receptors, Pex5p and Pex7p, bind their cognate peroxisomal targeting signals (PTS)[3] in the cytosol and then shuttle to peroxisomes, where the PTS proteins are imported. After releasing their cargo, the receptors recycle to the cytosol for additional rounds of import. Most of the peroxisomal matrix proteins possess one of two evolutionarily conserved PTS, the C-terminal PTS1 or the N-terminal PTS2, which are specifically recognized by Pex5p and Pex7p, respectively (2, 3). A few matrix proteins are known that contain a completely different targeting signal that is also recognized by Pex5p (9–12). This import receptor was shown to consist of two functionally distinct domains. Although binding of the PTS1 proteins is mediated by six tetratricopeptide repeats within its C-terminal half (13), essential transport steps of the receptor cycle seem to be performed by its N-terminal half (9, 14).

Numerous reports demonstrate that mammalian and yeast Pex5p tightly associate with peroxisomal membranes (15–20). Membrane peroxins that bind directly to both receptors, Pex5p and Pex7p, are Pex13p (21–24) and Pex14p (25–27). These two proteins together with Pex17p have been established as members of a membrane-bound docking subcomplex (28, 29). An even larger complex, termed the importomer, which in addition to the docking subcomplex contains Pex8p and the three RING finger peroxins, Pex2p, Pex10p, and Pex12p, has been shown to exist in the peroxisomal membrane (29). The ATP-dependent dislocation of the PTS1 receptor from the peroxisomal membrane into the cytosol is mediated by the AAA peroxins Pex1p and Pex6p (30). The interaction of Pex5p and Pex14p, which seems to be the initial binding partner of the receptors at the peroxisomal membrane, has been studied intensively (31–34). It was found that the N terminus (amino acid residues 1–78) of human Pex14p directly interacts with Pex5p with a binding affinity in the nanomolar range (32). Moreover, the seven conserved W*XXX*F motifs within the N-terminal half of human Pex5p form individual high affinity sites for Pex14p (35). These motifs are present in variable number and spacing in all known Pex5 proteins. Recently it was shown that the PTS1 and the PTS2 receptors not only associate with the outer surface of the membrane (cycling receptor model) but actually traverse the membrane. This has led to the extended shuttle

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We dedicate this manuscript to Helmut Kindl in honor of his 70th birthday and his contribution to the field of peroxisome biogenesis.

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[3] The abbreviations used are: PTS, peroxisomal targeting signal; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PMSF, phenylmethylsulfonyl fluoride; MES, 4-morpholineethanesulfonic acid; aa, amino acid; ProtA, Protein A.

TABLE 1
S. cerevisiae strains used

Strain	Description	Ref.
UTL-7A (wild-type)	*MATα, ura 3-52, trp1, leu2-3/11*	38
Wild-type Pex5p-ProtA	UTL-7A, *PEX5*-TEV-ProteinA-kanMX6-T_{pex5}	14
pex5Δ	*MATα, ura 3-52, trp1, leu2-3/112, pex5::LEU2*	14
pex5Δpex1Δ	*MATα, ura 3-52, trp1, leu2-3/112, pex1::loxP, pex5::loxPkan*	30
pex3Δpex19Δ	*MATα, ura 3-52, trp1, leu2-3/112, pex3::loxPkan, pex19::loxP*	This study
pex5Δpex13Δpex14Δ	*MATα, ura 3-52, trp1, leu2-3/112, pex5::loxP, pex13::loxP, pex14::loxPkan*	This study
PCY2	*Matα, D*gal4, *D*gal180, URA::*GAL1-lacZ, lys2–801*^{amber}, *his3-D*200, *trp1-D63, leu2 ade2-101*^{ochre}	39

receptor model (6). Despite these accumulating data about the translocation machinery, a translocation channel has not yet been identified.

In this study, we investigated the interaction of *Saccharomyces cerevisiae* Pex5p (*Sc*Pex5p) at the peroxisomal membrane in greater detail. The identification of a novel Pex14p-binding site in the Pex5p sequence enabled us to generate a mutant Pex5p that has lost the ability to associate stably with the docking and the RING finger complex. Most unexpectedly, this Pex5p variant associated together with cargo protein with the peroxisomal membrane and was still able to mediate matrix protein import. From these findings we speculated that protein-lipid interaction rather than protein-protein interactions could anchor the receptor-cargo complex at the membrane. In a first step to verify this assumption, we show *in vitro* that Pex5p has the ability to spontaneously insert into phospholipid monolayers and bilayers.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions—Yeast strains used in this study are derivatives of *S. cerevisiae* UTL-7A if not stated otherwise (Table 1). Strains expressing proteins fused to tobacco etch virus (tobacco etch virus-protease cleavage site)-protein A instead of wild-type Pex5p were generated by genomic integration into the PEX5 locus. This was achieved by transforming haploid yeast cells with the PCR products according to Knop *et al.* (36). The yeast strain *pex3Δpex19Δ* was generated according to Güldener *et al.* (37) using primer pairs Ku365/Ku700 (Table 2) for PEX19 deletion and Ku575/Ku862 for PEX3 deletion. The construction of the *pex13Δ pex14Δ pex5Δ* strain required the disruption of PEX5 with the removable *loxP-kanMX4-loxP* marker using the oligonucleotides Ku301 and Ku976b. The deletion of PEX13 was generated using the primer pair Ku274/RE534, whereas PEX14 was deleted with the oligonucleotides RE536 and RE537. Complete and minimal media used for yeast culturing have been described previously (38).

DNA Manipulations—All expression plasmids used in this study code for yeast proteins if no other organism is noted. All plasmids named pDK or pWK contain modified copies of the *ScPEX5* gene, which were expressed in a PEX5 deletion strain from the low copy vector pRS416 (Stratagene). The constructs are under control of the *PEX5* promotor region and an ADC1 termination region (14). The corresponding coding DNA regions were amplified by PCR using genomic DNA of the wild-type strain as a template. The oligonucleotides used in this study are listed in Table 2 and were obtained from Eurogentec (Belgium).

The PEX5 full-length fragment was amplified by using the primers Ku875 and Ku888. The amplification product was cloned using primer-generated endonuclease recognition sites into SalI/BglII-digested pWK-PEX5-(aa 1–313) (14) and into SalI/BglII-digested pPC86 (39) resulting in pWK-PEX5 and pPC86-PEX5. The PEX5-(aa 1–313) fragment was obtained by SalI/BglII digestion from the vector pWK-PEX5-(aa 1–313) (14) and ligated into a SalI/BglII-digested two-hybrid vector pPC86 resulting in pPC86-PEX5-(aa 1–313). The same procedure was used to obtain the PEX5-(aa 313–612) fragment from the vector pDK-PEX5-(aa 313–612) (14) resulting in pPC86-PEX5-(aa 313–612). The PEX5-(aa 1–245) fragment was amplified using the primer pair Ku875/Ku876. The amplification product was digested with SalI and BglII and ligated into a SalI/BglII-digested pPC86 vector resulting in pPC86-PEX5-(aa 1–245). The PEX5 fragment encoding amino acids 246–267 was amplified by two PCRs. In a first step a fragment corresponding to amino acids 246–258 fused to the GAL-4-activating domain was amplified using the two-hybrid vector pPC86 as template and the primer pair Ku1436/Ku1433 in which the latter one codes for the PEX5 fragment. The PCR product serves as template for the final fragment coding for amino acids 246–267, which was amplified by the primer pair Ku1436/Ku1434 in which the latter one codes for the PEX5 fragment (aa 252–267) and contains a NotI restriction site. The amplification product was ligated after MluI/NotI restriction into an MluI/NotI-digested pPC86 vector resulting in pPC86-PEX5-(aa 246–267).

Point mutations in PEX5 were introduced using overlap extension PCR (40). All base pair changes were verified by sequencing. For the mutation Pex5p(W120A), PCR products were amplified by primer pairs Ku875/Ku856 and Ku855/Ku888 using genomic DNA of wild-type strain as template. Ku856 and Ku855 contain a substituted base pair triplet coding for alanine at amino acid position 120. The exterior primers Ku875 and Ku888 together with both PCR products were used for the overlap extension PCR. The amplification product was digested with SalI and BglII and ligated into SalI/BglII-digested pPC86 resulting in pPC86-PEX5(W120A). To introduce the double mutation W120A/W204A into the PEX5 sequence, the PCR product PEX5(W120A) was used as a template. Overlapping PCR was carried out as described above using primers Ku857 and Ku858 for the substitution W204A and the exterior primers Ku875 and Ku888. The PCR product was digested with SalI and BglII and ligated into SalI/BglII digested pPC86 resulting in pPC86-PEX5(W120A;W204A). To introduce the single mutation Pex5p(Trp-204) the same cloning procedure was carried out as described above but using genomic DNA of wild-type strain as template resulting in pPC86-PEX5(W204A).

TABLE 2
Oligonucleotides used

Designation	Sequence (5′ to 3′)
KU 274	TATCTATAAATATCAAGGGGATTCTATACTATAACAATACCTGCGCGTACGCTGCAGGTCGAC
KU 301	TATACATCAATAAACAATATATCATAACACATGGACGTACGTACGCTGCAGGTCGAC
KU 365	AAGAATTACAAATTGTGGGAACCGAAGTATTGACGGAAAGAAGAAAT
KU 575	CGTAAAAGCAGAAGCACGAAACAAGGAGGCAAACCACTAAAAGGCGTACGCTGCAGGTCGAC
KU 862	CATGTTACCTATTGCACACTTACTGTATTAAAGATTACGCATAGGCCACTAGTGGATCTG
KU 700	TACTTTTTTTTTTTTTTTTTTACTGTTATCATAAATATATATACCTTAATAGGCCACTAGTGGATCTG
KU 855	TATATCTCATGCTTCACACAGGAATTTCAAGGTAGTA
KU 856	TCCTGTGAAGCATGAGATATATCGTTCACTCC
KU 857	CAACCCGCTACAGATCAGTTTGAAAAGCTG
KU 858	CTGATCTGAGCGGGTTGTTGTTCTTGCTCATT
KU 875	TCAGTGTCACCATGGACGTAGGAAGTTGCTCA
KU 876	CAGAGATCTTCATTCCTTCTCAACAGTTTCTGG
KU 888	AGAGATCTTCAAAACGAAAATTCTCCTTTAAATC
KU 976b	TGATGCGAGAACATAAAATTGCGGAGAACCATATCAATAGGCCACTAGTGGATCTG
KU 1457	AAGAAGTGGCGGATAGCATACACAAGGACG
KU 1458	TATCCGCCACTTCTTGGAAATCAGATTGA
KU 1436	CTATACCAAGCATACAATCAAC
KU 1433	TTGGAAATCAGATTGATACTGATCTCCATATACTCCTTCCTGAATTCCCGGGGTCGACC
KU 1434	GATGTGCGGCCGCGTCCTTGTGTATGCTATCCCACACTTCTTGGAAATCAGATTGATAGTGA
KU 1520	CGAAAGCTTATGAAGCTACTGTCTTCTATCG
KU 1521	AATGCGGCCGCTCATAACTTTGCTTTTCGTTTATTCTCATTTTCCAAGGCGTAGATCTGAATTCCCGGGG
KU 1584	ACTCGGCATGCAACAGATCTATATTACCCTGTTATC
RE 534	CTAGTGTGTACGCGTTTCATCATCAACATGCTCAATTTTCTTCCGATAGGCCACTAGTGGATCTG
RE 536	TTTGAAAACTCAAGTAAAACAGAGAAGTTGTAAGGTGAATAAGGACAGCTGAAGCTTCGTACGCT
RE 537	CTATGGGATGGAGTCTTCGACCTGTCCATTTTGCCAGTCAGGGACATAGGCCACTAGTGGATCTG

Additionally, the PCR fragment was ligated into SalI/BglII-digested pWK-PEX5-(aa 1–313) resulting in pDK-PEX5(W204A). To obtain Pex5p(W261A), PCR products were amplified by primer pairs Ku875/Ku1458 and Ku888/Ku1457 using genomic DNA of wild-type strain as template. Ku1458 and Ku1457 contain a substituted base pair triplet coding for alanine at amino acid position 261. The exterior primers Ku875 and Ku888 together with both PCR products were used for the overlap extension PCR. The amplification product was digested with SalI and BglII and ligated into SalI/BglII-digested pWK-PEX5-(aa 1–313) (14) and into SalI/BglII-digested pPC86 (39), resulting in pDK-PEX5(W261A) and pPC86-PEX5(W261A). To introduce the double mutation W120A/W204A into the PEX5 sequence, PEX5(W204A) was used as a template. Overlapping PCR was carried out as described above using primers Ku1558 and Ku1457 for the substitution W204A and the exterior primers Ku875 and Ku888. The amplification product was digested with SalI and BglII and ligated into SalI/BglII digested pPC86 and pWK-PEX5-(aa 1–313) resulting in pPC86-PEX5(W204A;W261A) and pDK-PEX5(W204A;W261A).

The PTS1 peptide represents the 12 C-terminal amino acids 659–670 from the carnitine-acetyltransferase 2. The fragment was amplified by the primer pair Ku1520/Ku1521 using the two-hybrid vector pPC97 as template. The primer Ku1520 contains a HindIII restriction site and binds on the Gal-4 binding domain of the vector. The primer Ku1521 contains a NotI restriction site and encodes for the PTS1 sequence. The amplification product was ligated after HindIII/NotI restriction into HindIII/NotI-digested pPC97 vector resulting in pPC97-PTS1.

The vectors pPC97-PEX14-(aa 1–58) and pPC97-PEX14-(aa 235–341) were kindly provided by K. Niederhoff (Bochum, Germany) (41). Plasmid pPC97-PEX14 was described by Albertini *et al.* (42). The vector pPC97-PEX8 was described by Rehling and co-workers (40, 43). The vector pPC97-PEX13 harbors a PEX13 fragment that encodes the Src homology 3 domain ranging from amino acid positions 286 to 386 (24).

To create yeast expression plasmids coding for Pex5p(W261A)-ProtA, Pex5p(W204A)-ProtA, and Pex5p(W204;261A)-ProtA, a PCR product containing a PEX5-ProtA fragment (14) was obtained with the primer pair Ku875 and Ku1584 (containing a SpHI restriction site) by using genomic DNA isolated from strain UTL-Pex5p-ProtA as a template. The amplification product was cloned after NheI/SpHI restriction into NheI/SpHI-digested pWK-Pex5p(W261A), pWK-Pex5p(W204A), and pWK-Pex5p(W204;261A), resulting in pDK-Pex5p(W261A)ProtA, pDK-Pex5p(W204A)ProtA and pDK-Pex5p(W204;261A)ProtA.

For bacterial expression of yeast Pex5p, the vector pET9d-His-*Sc*Pex5p was kindly provided by K. Niederhoff (Bochum, Germany). For expression of human Pex5p, the vector pET9d-His-*Hs*Pex5Lp was used (32).

Two-hybrid Assay—For two-hybrid assays based on the method of Fields and Song (44), open reading frames of selected PEX genes were fused to the DNA-binding domain or transcription-activating domain of GAL4 in the vectors pPC86 and pPC97 (39). Cotransformation of two-hybrid vectors into yeast strain PCY2 (Clontech) was performed according to the protocols of the manufacturer. Transformed yeast cells were plated on SD synthetic medium without tryptophan and leucine. β-Galactosidase filter tests were performed as described previously (45).

Western Blotting and Densitometry—Western blots were incubated with polyclonal rabbit antibodies raised against human Pex5p and the *S. cerevisiae* proteins thiolase, Fox1p, Pex5p, Pex10p, Pex12p, Pex13p, Pex14p, Pex17p, Pex3p, catalase A, porin, fructose-1,6-bisphosphatase (all raised in our laboratory), aconitase (a kind gift of R. Lill, University of Marburg, Germany), Mdh3p (a kind gift of L. McAlister-Henn, University of California), and Sec72p (a kind gift of E. Hartmann, University of Lübeck, Germany). Horseradish peroxidase coupled with anti-rabbit IgG in combination with the ECL system (Amersham Biosciences) was used to detect immunoreactive complexes.

For semi-quantitative analyses of Western blot signals the band density on film was measured with a scanner using Scion image software. The relative density of signals was calculated in the area encompassing the immunoreactive protein band and subtracting the background of an adjacent nonreactive area in the same lane of the protein of interest.

Yeast Cell Fractionation—Spheroplasting of yeast cells, homogenization, and differential centrifugation at 25,000 × *g* of post-nuclear supernatants were performed as described previously (38). Cell fractionation by means of density gradient centrifugation was carried out as described previously (14), but instead of postnuclear supernatant 3–4 mg of protein of 25,000 × *g* organellar pellet were loaded onto the gradient. For membrane extraction of organellar pellets, aliquots were adjusted to a final concentration of 0.1 M Na_2CO_3, pH 11.5, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were incubated for 30 min on ice and subjected to centrifugation at 100,000 × *g* for 1 h. Protease protection experiments were carried out with organellar pellets containing 100 μg of protein and increasing amounts of proteinase K (Sigma) in buffer (0.6 M sorbitol, 5 mM MES, 0.5 mM EDTA, 50 mM KCl, pH 6.0) with or without 1% Triton X-100. Digestion was carried out on ice for 10 min and stopped by adding PMSF (240 μg/ml) and SDS-PAGE sample buffer. All samples were analyzed by Western blotting.

For flotation analyses of cell lysates, oleate grown yeast cells were lysed according to Lamb *et al.* (46) using glass beads and lysis buffer (20 mM HEPES; 100 mM KOAc; 5 mM MgOAc; pH 7.5) containing protease inhibitors (240 μg/ml PMSF, 2 μg/ml aprotinin, 0.35 μg/ml bestatin, 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, 0.16 mg/ml benzamidine, 5 μg/ml antipain, 0.21 mg/ml NaF, 6 μg/ml chymostatin). 1.5 mg of protein from the lysate were adjusted to a concentration of 45% (w/v) sucrose. The samples were laid onto sucrose cushions (220 μl of lysis buffer with 50% (w/v) sucrose) and overlaid with 500 μl of buffer II (lysis buffer; 40% (w/v) sucrose), 1900 μl of buffer I (lysis buffer; 25% (w/v) sucrose), and 1000 μl of lysis buffer. After ultracentrifugation for 3 h at 170,000 × *g* in a swing-out rotor, the flotation gradient was collected as 10 fractions from top (fraction 10) to bottom (fraction 1). The fractions were analyzed by SDS-PAGE and Western blot detection.

The immunopurification of membrane-associated Pex5p-ProtA and its variants with IgG-coupled Sepharose was performed as described previously (14, 29). For densitometric analyses of Western blot signals, at least two independent experiments and various exposure times were used. During the course of all fractionation experiments, SDS-PAGE samples of homogenates and lysates were taken immediately after cell breakage.

Electron Microscopy—For electron microscopy, oleate-induced cells were fixed with 1.5% $KMnO_4$ and prepared as described by Erdmann *et al.* (38).

Expression, Purification, and Analysis of Recombinant Proteins—Expression and purification of His_6-tagged human and yeast Pex5p were carried out as described previously (32) with slight modifications. After binding to the nickel-nitrilotriacetic acid matrix (Qiagen, Germany), yeast proteins were eluted with 100 mM imidazole, and dithiothreitol was added to the eluate to a final concentration of 10 mM.

In Vitro Assays—The membrane insertion experiments into lipid monolayers and liposomes were carried out with mixtures of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), purchased from Avanti Polar Lipids, Inc., and stock solutions of lipids were prepared in chloroform. The phospholipid phosphorus concentration was determined using the method of Rouser *et al.* (47). The final lipid mixtures were made at a concentration of 10 mM.

Lipid monolayers were spread on subphase buffer of 20 mM Tris-HCl, pH 7.4, 50 mM NaCl (continuously stirred with a magnetic bar) to give an initial surface pressure of 20 mN/m. Proteins were injected through a hole at the side of the monolayer trough (volume 5 ml); the injection volume was less than 1% of the volume of the subphase. The surface pressure of the monolayer was measured by the Wilhelmy plate method, using a Cahn 2000 electrobalance (48). Experiments were performed in a thermostatically controlled cabinet at room temperature.

For liposome preparation DOPC and DOPE were mixed together at a molar ratio of 7:3 in chloroform. The organic solvent was removed by placing the flask on a rotary evaporator (Buchi Rotavapor, Switzerland) operating at 80 rpm under a low vacuum at 40 °C, until a thin lipid film was produced on the bottom. The dried lipid film was hydrated in 20 mM Tris, 50 mM NaCl, pH 8, to a final concentration of 10 mM. Small unilamellar vesicles were obtained by sonication of the multilamellar vesicle suspension using a Branson sonifier (Branson Ultrasonic Corp.). The procedure was continued at room temperature until the solution became clear. The vesicles were stored at 4 °C and used within 24 h.

Purified human and *S. cerevisiae* Pex5p (0.75 nmol) were incubated for 1 h at room temperature in the absence or presence of liposomes (ratio of 1:750) in a final volume of 80 μl and mixed with 170 μl of buffer III (20 mM Tris-HCl; 50 mM NaCl; 65% (w/v) sucrose, pH 7.4). The samples were transferred to 4-ml ultracentrifuge tubes onto a sucrose cushion (220 μl of buffer III) and overlaid with 500 μl of buffer II (20 mM Tris-HCl; 50 mM NaCl; 40% (w/v) sucrose, pH 7.4), 1900 μl of buffer I (20 mM Tris-HCl; 50 mM NaCl; 25% (w/v) sucrose, pH 7.4), and 1 ml of buffer 0 (20 mM Tris-HCl; 50 mM NaCl, pH 7.4). After ultracentrifugation for 3 h at 170,000 × *g* in a swing-out rotor, the gradient was collected as 10 fractions from top to bottom. The fractions were analyzed by SDS-PAGE and Western blot detection.

For protease protection assays, floated fractions from gradients containing Pex5p were mixed with trypsin (Sigma) in a 60:1 ratio (according to Western blot signals). As a control, the corresponding load fractions were used. Protease treatment was carried out on ice. Trypsin was inactivated by incubation with SDS sample buffer for 5 min at 95 °C. Samples were taken after 0, 0.5, 1, 3, 5, and 10 min and analyzed by SDS-PAGE and immunoblotting.

In Vitro Export—In vitro import/export studies were performed as described previously (30).

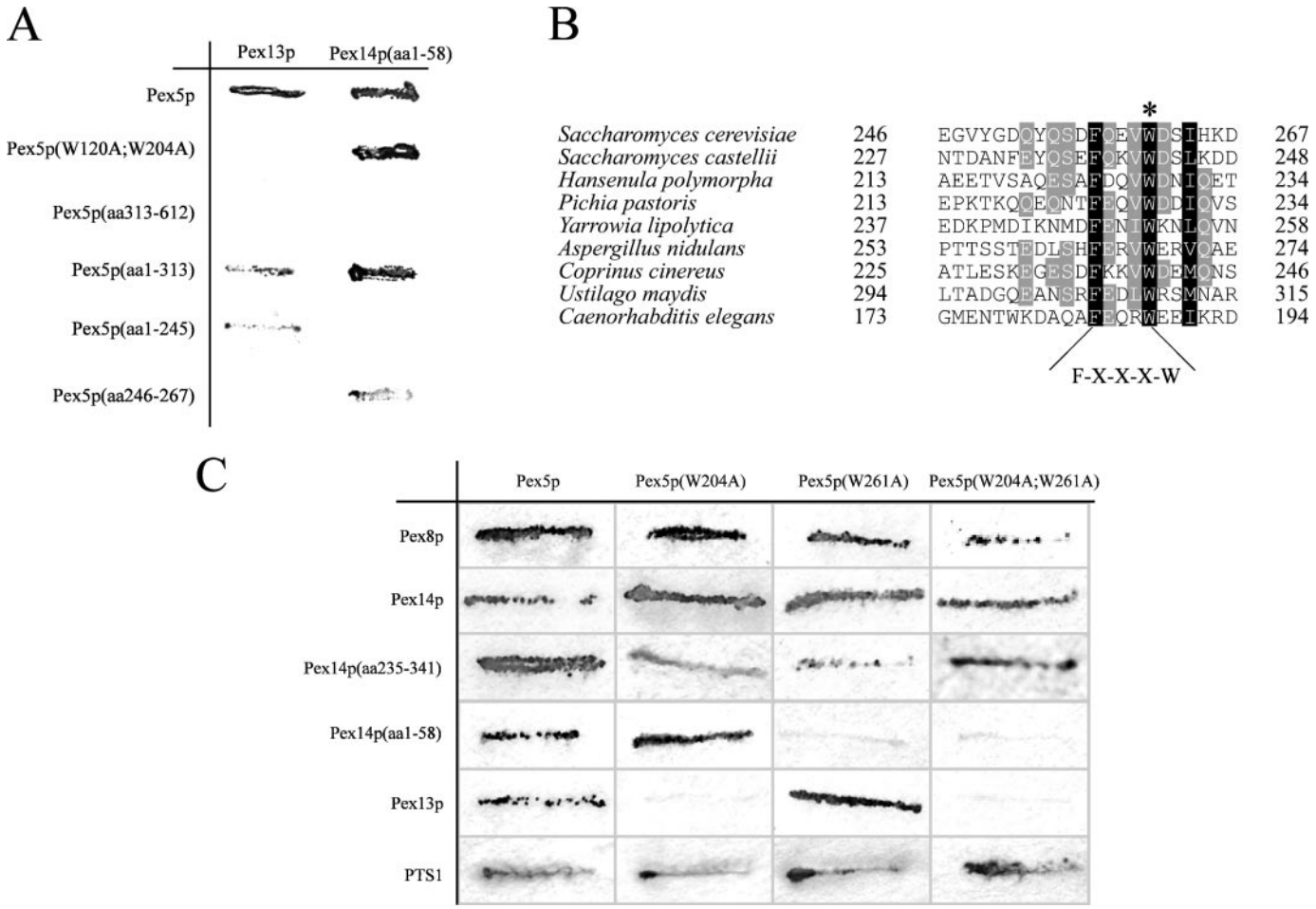

FIGURE 1. **Pex5p-binding site for the conserved N-terminal domain of Pex14p contains an inverted W*XXX*F motif.** *A*, Pex5p fragment consisting of amino acids 246–267 is sufficient to bind the N-terminal domain of Pex14p (amino acids 1–58). For the two-hybrid assay Pex5p and truncations thereof were fused to the Gal4 binding domain and coexpressed with Gal4 activation domain fused to either Pex13p or Pex14p in yeast strain PCY2. β-Galactosidase activity was analyzed with a filter assay after selection on SD plates lacking leucine and tryptophan. *B*, an inverted W*XXX*F motif is conserved among Pex5p sequences of fungi and *C. elegans*. Sequences were aligned using ClustalX. *Black boxes* denote 100% sequence conservation, and *gray boxes* indicate more than 50% conserved amino acid residues. The *asterisk* marks the position of the conserved tryptophan, Trp-261, in *S. cerevisiae* Pex5p sequence. F*XXX*W indicates the inverted W*XXX*F motif. *C*, a single point mutation, W261A, within the Pex5p sequence abolishes binding to Pex14p-(aa 1–58). The indicated mutations W204A and W261A were introduced into the two-hybrid plasmid pPC86-PEX5 and transformed together with any of the following plasmids: pPC97-PEX8, pPC97-PEX13(SH3), pPC97-PEX14, pPC97-PEX14-(aa 1–58), pPC97-PEX14-(aa 225–341), and pPC97-PTS1. Selected transformants were analyzed for β-galactosidase activity with a filter assay.

RESULTS

Identification of the Pex5p-binding Site for the Conserved N-terminal Domain of Pex14p in S. cerevisiae—Although accumulated evidence demonstrates that the PTS1 receptor Pex5p binds to several peroxins organized in multisubunit complexes at the peroxisomal membrane (2), studies in a PEX8 deletion strain of *S. cerevisiae* strongly suggested that the initial association occurs with the docking subcomplex consisting of Pex13p, Pex14p, and Pex17p (29). To investigate the association of the receptor-cargo complex with the peroxisomal membrane in more detail, we mapped the Pex14p-binding site on Pex5p. Previous studies in higher eukaryotes demonstrated that the conserved N-terminal region of the membrane-associated Pex14p, referred to as Pex14p-N, interacts with the PTS1 receptor Pex5p via di-aromatic pentapeptide repeats (31, 33–35). *Sc*Pex5p possesses two of these W*XXX*F motifs, one of them is part of its Pex13p-binding site (21). We substituted the conserved tryptophans in both motifs of *Sc*Pex5p with alanine and tested the two-hybrid interaction with Pex13p and Pex14p-N. In line with previous reports, the W204A mutation abolished the interaction between Pex5p and Pex13p. However, neither of the two W*XXX*F motifs was required for binding of Pex14p-N (Fig. 1*A*).

To map the Pex14p-N-binding sites within the sequence of *Sc*Pex5p, we generated a number of truncated Pex5p versions and examined the fragments for their two-hybrid interaction with Pex14p-N (Fig. 1*A*). The smallest fragment in our analysis which retained interaction with Pex14p-N was located between amino acid positions 246 and 267. One striking feature within this stretch of amino acids is a di-aromatic pentapeptide that represents an inverted W*XXX*F motif. The sequence FQEVW is highly conserved among the Pex5p sequences from fungi and is also found in *Caenorhabditis elegans* Pex5p (Fig. 1*B*).

The significance of these conserved amino acid residues for Pex5p-Pex14p interaction was confirmed by site-directed mutagenesis and again two-hybrid analysis. The substitution of

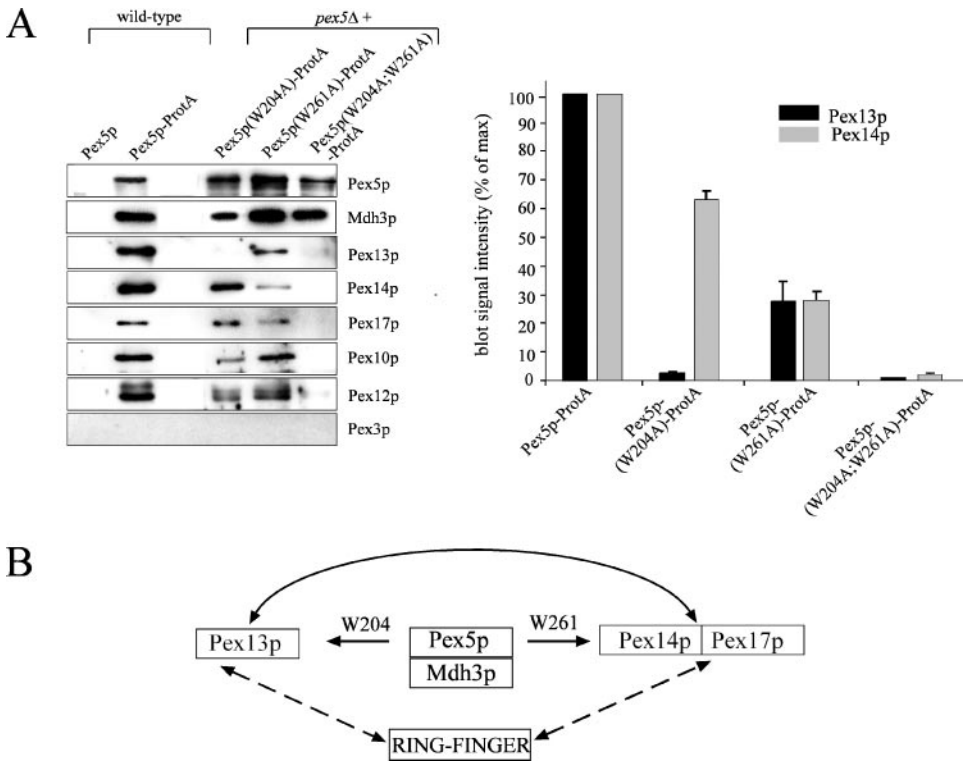

FIGURE 2. **Membrane-associated Pex5p(W204A;W261A) does not form stable complexes together with the docking and the RING finger peroxins.** *A*, the point mutations W204A and W261A alone or in combination affect the association of Pex5p with the docking and the RING finger complex. *S. cerevisiae* wild-type cells, in which the genomic PEX5 open reading frame was replaced by a DNA region coding for Pex5p-ProtA, *pex5*Δ cells expressing Pex5p(W204A)-ProtA, Pex5p(W261A)-ProtA, and Pex5p(W204A;W261A)-ProtA from plasmids and as a control for wild-type cells (Pex5p), were grown on oleate medium. Protein complexes were isolated from equal amounts of 1% (w/v) digitonin-solubilized membranes subjected to IgG affinity chromatography. Bound proteins were eluted with SDS gel electrophoresis sample buffer and subjected to immunoblot analysis with antisera directed against Pex5p, Mdh3p, Pex13p, Pex14p, Pex17p, Pex10p, Pex12p, and Pex3p (*left panel*). Sample volumes correspond to equal amounts of the indicated bait proteins. The *right panel* displays semi-quantitative densitometric analysis of Western blot signals obtained for Pex5p-associated Pex13p and Pex14p given in % of maximum (amount of protein bound to Pex5p-ProtA). The results presented are representative of two independent IgG-Sepharose purifications and various exposition times. Standard errors are indicated by *bars*. *B,* schematic representation of interactions between the ScPex5p-cargo complex and membrane peroxins as deduced from the experiment shown above.

the tryptophan 261 with alanine completely inhibited the interaction between Pex5p and Pex14p-N (Fig. 1*C*).

In contrast, this mutation did not impair the interaction of the PTS1 receptor with Pex8p, Pex13p, and PTS1 peptide indicating that the Pex5p variant is folded correctly (Fig. 1*C*). Therefore, we conclude that the ScPex5p fragment consisting of amino acids 246–267 and containing the inverted W*XXX*F motif FQEVW is sufficient and necessary for the interaction between Pex5p and the conserved N-terminal domain of Pex14p. However, Pex5p(W261A) still interacts with full-length Pex14p and with a C-terminal fragment of Pex14p consisting of amino acids 235–341 in the two-hybrid assay (Fig. 1*C*). It has been shown recently that Pex14p contains an additional binding site for the PTS1 receptor within the C-terminal domain of Pex14p (41, 49). Nonetheless, our data clearly prove that the Pex14p-C-binding site of Pex5p, which has not been mapped so far, is not identical with the inverted W*XXX*F motif.

Disruption of the Interaction of Cargo-laden Pex5p with the Docking and the RING Finger Complex—As a next step, we studied the effect of the two point mutations, W204A and W261A, in *Sc*Pex5p on the association of the PTS1 receptor with the peroxisomal membrane and, in particular, with constituents of the docking and the RING finger complexes. As shown above, these point mutations specifically disrupt the interactions of the PTS1 receptor with Pex13p and the N-terminal domain of Pex14p, respectively. The mutations were introduced in Pex5-Protein A (ProtA) expression plasmids, and extracts of digitonin-solubilized membranes of cells expressing the mutant Pex5p-ProtA fusion proteins were applied to affinity purification using IgG-Sepharose columns. We have demonstrated previously that the C-terminal ProtA tag does not affect the functionality of the PTS1 receptor (14). Fig. 2*A* (*2nd lane*) shows in accordance with previous results that affinity-purified fractions using Pex5p-ProtA as a bait contain Pex13p, Pex14p, Pex17p, as well as the RING finger peroxins Pex10p and Pex12p. As a negative control, Pex3p was not found associated with Pex5p. It is important to note that in addition to known binding partners of Pex5p at the membrane, malate dehydrogenase 3 (Mdh3p) could be detected in all eluates of the IgG-Sepharose column. This soluble peroxisomal enzyme had been reported to be one of the most abundant PTS1-containing matrix proteins in cells of *S. cerevisiae* after oleate induction (50, 51). It is very likely that membrane-associated Mdh3p represents a cargo on its way into the peroxisome. Thus, the presence of Mdh3p in all eluates indicated the ability of the tested Pex5p variants to bind PTS1 proteins and to target their cargo to the peroxisomal membrane (Fig. 2*A, 2nd* to *5th lanes*). Specific disruption of the Pex5p-Pex14p-N interaction by the single W261A mutation only slightly reduces the amounts of Pex5p-associated Pex13p, Pex14p, Pex17p, Pex10p, and Pex12p (Fig. 2*A*, *4th lane*). The Pex5p variant harboring only W204A, in agreement with the two-hybrid results, led to affinity-purified eluates that selectively lacked Pex13p (Fig. 2*A*, *3rd lane*) but still contain Pex14p, Pex17p, Pex10p, and Pex12p. In contrast, in eluates obtained from cells expressing the Protein A-tagged Pex5p with both mutations not any component of the docking complex was detected. The lack of an interaction with Pex17p is probably due to the fact that Pex5p interacts with Pex17p indirectly through Pex14p. Moreover, the components of the RING finger subcomplex were not found. To test the absence of binding partners of Pex5p(W204A;W261A) more rigorously, Western blots obtained from two independent affinity purifications were subjected to prolonged exposure

times. Therefore, traces of Pex13p and Pex14p but still no RING finger peroxins were found (Fig. 2*A*, *right panel*). Taking into account the known interaction between Pex13p and Pex14p (21, 42, 52), we can deduce from our results a complex network of interactions between Pex5p-cargo and constituents of the docking and RING finger complexes as shown schematically in Fig. 2*B*. Our data strongly suggest that either Pex13p or Pex14p-N is required to associate Pex5p stably to peroxins acting downstream of the docking event.

Pex5p(W204A;W261A) Mediates PTS1 Protein Import into Peroxisomes—In order to study the effect the uncoupling of Pex5p from the docking complex has on peroxisome protein import, the mutant Pex5 proteins, Pex5p(W204A), Pex5p(W261A), Pex5p(W204A;W261A), and the corresponding Protein A-tagged variants were expressed in a PEX5 deletion strain. Surprisingly, all tested variants of Pex5p are able to partially complement the oleate growth defect of *pex5*Δ cells as indicated by the oleate plate drop dilution assay (Fig. 3*A*, *left panel*) or growth in liquid oleate medium (Fig. 3*A*, *right panel*). The unexpected ability of these mutants to facilitate import of peroxisomal matrix proteins is further substantiated by electron microscopy and subcellular fractionation. Electron micrographs of cells expressing these mutated Pex5 proteins revealed the presence of membrane-bound organelles that resemble in size and number wild-type peroxisomes (Fig. 3*B*). Subcellular fractionation of cells expressing Pex5p(W204A;W261A) by means of 25,000 × *g* centrifugation (Fig. 3*C*) clearly showed that the import of the PTS1 proteins catalase and Mdh3p as that of the PTS2 protein thiolase into peroxisomes is not drastically affected. In contrast, acyl-CoA oxidase (Fox1p) is found mostly in the 25,000 × *g* supernatant suggesting a cytosolic mislocalization. This import defect is consistent with recent observations of Klein *et al.* (10). The authors demonstrated that the W261A mutation drastically weakens binding efficiency of Pex5p with acyl-CoA oxidase and peroxisomal carnitine acetyltransferase, both of which utilize unusual non-PTS1 non-PTS2 targeting signals. Therefore, it seems likely that the reduced growth rates of the W261A single and double mutants on oleate compared with wild-type Pex5p (Fig. 3*A*) are due to lower binding efficiency of this specific subset of cargo proteins rather than to a general failure of this mutant to facilitate import of peroxisomal proteins.

25,000 × *g* sediments obtained from *pex5*Δ cells complemented with Pex5p(W204A;W261A) were further subjected to density gradient centrifugation (Fig. 3*D*). Peroxisomal matrix and membrane proteins cofractionated at a density of 1.16 g/cm^3, whereas wild type peroxisomes were found at a density of 1.17 g/cm^3. This difference could be explained by the partial import defect caused by the W261A mutation. In line with the results of the functional complementation analysis, Pex5p(W204A;W261A) is clearly associated with the peroxisomal fraction. However, scanning densitometric analysis of Pex5p Western blot signals in both gradients revealed that the amount of Pex5p(W204A;W261A) associated with mature, intact peroxisomes (17.8%) is significantly less than wild-type Pex5p (31.8%). A major portion of Pex5p(W204A;W261A) seems to be associated also with other cellular organelles, probably mitochondria. Therefore, disruption of the binding sites for Pex14p and Pex13p results in more unspecific binding of the PTS1 receptor to other membranes.

In summary, the wild-type like morphology of peroxisomes (Fig. 3*B*) and the relative amounts of PTS1 matrix enzymes associated with these organelles (Fig. 3, *C* and *D*) in the double mutant cells clearly support the notion that the Pex5p(W204A;W261A) variant is able to target PTS1 proteins to peroxisomes and to facilitate their translocation across the peroxisomal membrane.

Membrane Binding of Pex5p Does Not Require a Stable Association with the Docking and the RING Finger Complex—Next we asked whether disruption of interacting sites affects the membrane binding properties of the PTS1 receptor. To quantify the amounts of Pex5p associated with membranes, oleate-induced cells were disrupted with glass beads, and the resulting homogenates were subjected to flotation analyses. Both Pex5p(W204A;W261A) and its Protein A-tagged variant cofractionate like wild-type Pex5p in light density regions of the gradients together with typical membrane marker proteins for peroxisomes, mitochondria, and endoplasmic reticulum (Fig. 4*A*). Remarkably, the amount of membrane-associated Pex5p(W204A;W261A), which barely binds to other membrane proteins, is comparable with that of wild-type Pex5p. In light of these findings, we conclude that the interactions of Pex5p either with Pex13p or with Pex14p-N, which are required to connect the PTS1 receptor with the docking and the RING finger complex, are not necessary for membrane attachment of cargo-laden Pex5p.

In order to test whether mutant Pex5p binds exclusively to peroxisomal membranes, we used the double deletion strain *pex3*Δ*pex19*Δ, which should not contain peroxisomal membranes at all (53). Unexpectedly, a small fraction of the PTS1 receptor and higher amounts of both mutant forms were found in the floated membrane fraction indicating unspecific binding to other cellular vesicles (Fig. 4*A*) in the absence of peroxisomal membranes.

In order to study the influence of Pex14p and Pex13p binding on the membrane topology of Pex5p, we applied carbonate extraction and proteinase K treatment to 25,000 × *g* organellar pellets obtained from wild-type cells and from *pex5*Δ cells expressing Pex5p(W204A;W261A). In contrast to integral membrane proteins like Pex3p, both Pex5p proteins, wild-type and double mutant, seem to exist in various membrane-bound states. Although most of the organellar Pex5 proteins were sensitive to carbonate extraction (Fig. 4*B*) and proteinase K digestion (Fig. 4*C*), small fractions of both proteins were completely resistant against these treatments. A possible interpretation of these results is that a large fraction of organellar Pex5p is more loosely associated with the membrane, most likely the cytosolic side, whereas a minor portion is intrinsically embedded in the membrane. Apparently, the mutant Pex5p(W204A;W261A) that is not stably associated with other peroxins exhibits the same membrane binding properties as wild-type Pex5p.

Membrane-associated Pex5p(W204A;W261A) Can Shuttle Back to the Cytosol in a Pex1p- and ATP-dependent Manner—In order to test whether the impairment to associate stably with the importomer affects recycling of the PTS1 receptor, we performed *in vitro* import/export studies with the Pex5p

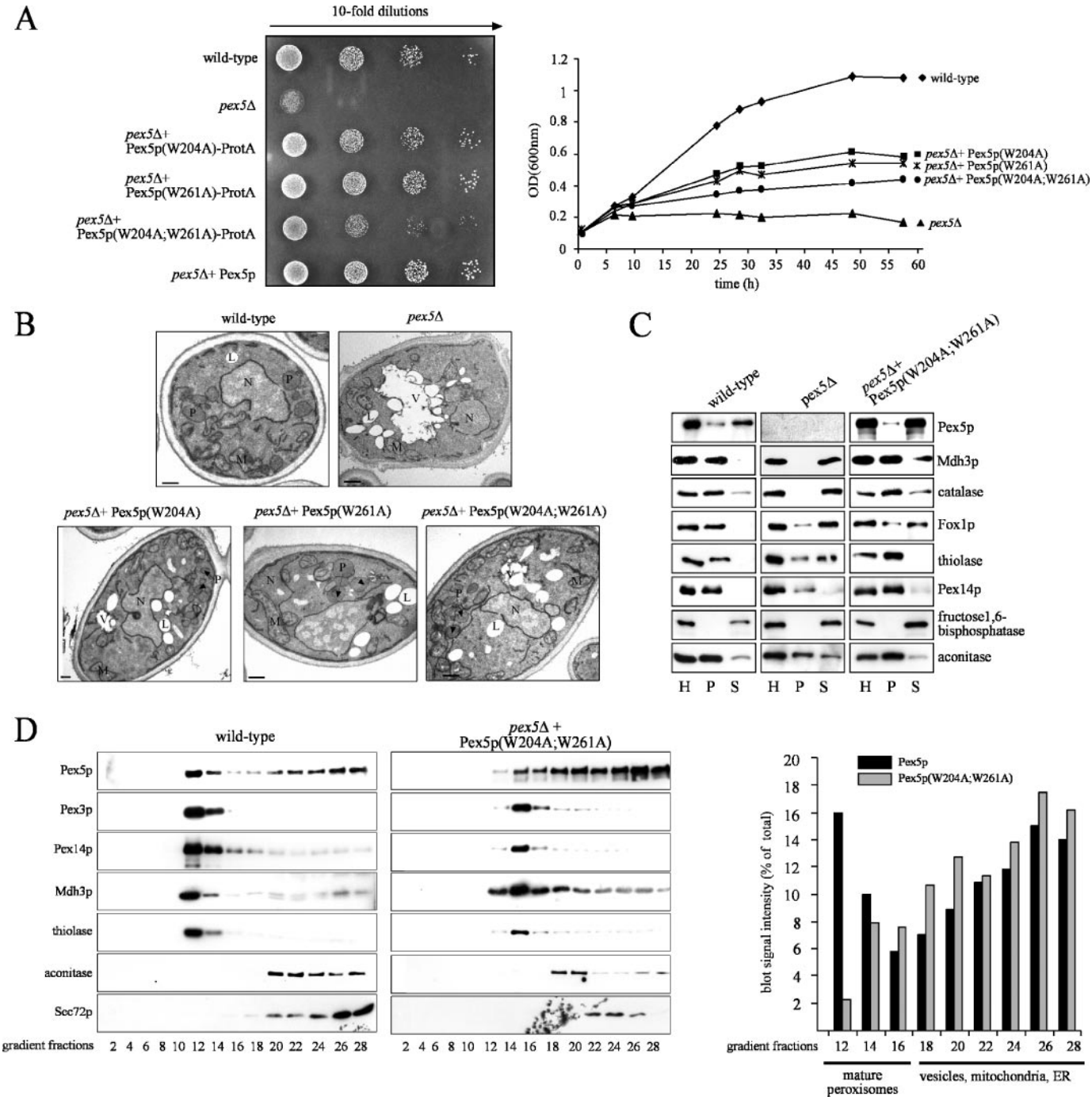

FIGURE 3. **The Pex5p variants Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) can mediate the import of PTS1 proteins into peroxisomes.** *A*, expression of the Pex5p variants Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) under control of the endogenous promoter partially rescue the PTS1 import defect in *pex5*Δ cells. For the plate growth assay (*left panel*), *pex5*Δ cells expressing Pex5p(W204A)-ProtA, Pex5p(W261A)-ProtA, and Pex5p(W204A;W261A)-ProtA were grown in 0.3% glucose medium for 16 h. Cells were washed and diluted to 3×10^7 cells/ml in distilled water. Aliquots were applied as a series of 10-fold dilutions onto an oleic acid plate, whereas the first spots on the left side correspond to 2×10^4 cells. The growth plates were incubated at 30 °C for 5 days. For the growth curves (*right panel*) cells expressing Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) were grown to mid-log phase in 0.3% glucose medium and inoculated at A_{600} of 0.1 in liquid oleate medium. Growth was followed with time by measuring the absorbance at 600 nm for 60 h. *B*, peroxisomes of cells expressing the mutated Pex5p variants exhibit wild-type like morphology. Electron microscopy analysis of wild-type cells, *pex5*Δ cells, and *pex5*Δ cells expressing Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) were carried out with cells grown for 12 h in oleic acid medium. *L*, lipid drops; *M*, mitochondria; *N*, nucleus; *V*, vacuole; *P*, peroxisomes; *Bars*, 1 μm. *C*, wild-type cells, *pex5*Δ cells, and *pex5*Δ cells expressing Pex5p(W204A;W261A) were grown for 14 h on oleate and subjected to subcellular fractionation. Equivalent volumes of the 600 × *g* postnuclear supernatant (*H*), 25,000 × *g* sediment (*P*), and 25,000 × *g* supernatant (*S*) were analyzed by Western blotting. Antibodies used were directed against Pex5p, Mdh3p, catalase A, Fox1p, thiolase, Pex14p, cytosolic fructose-1,6-bisphosphatase and mitochondrial aconitase. *D*, oleate-grown *pex5*Δ cells expressing Pex5p(W261A;W204A) and wild-type cells were further analyzed by density gradient centrifugation. 25,000 × *g* sediments were loaded on top of linear Optiprep gradients (15.5 to 36% (w/v) Iodixanol) containing 18% (w/v) sucrose and subjected to centrifugation. Fractions were collected from the bottom (fraction 1) of the gradient. Equal quantities of each second gradient fraction were analyzed by Western blotting using antibodies directed against Pex5p, Pex14p, Pex3p, Mdh3p, thiolase, aconitase, and Sec72p (*left panels*). The *right panel* displays the corresponding densitometric analyses of anti-Pex5p Western blot signals. Results are given in percent of the sum of all signals in each gradient.

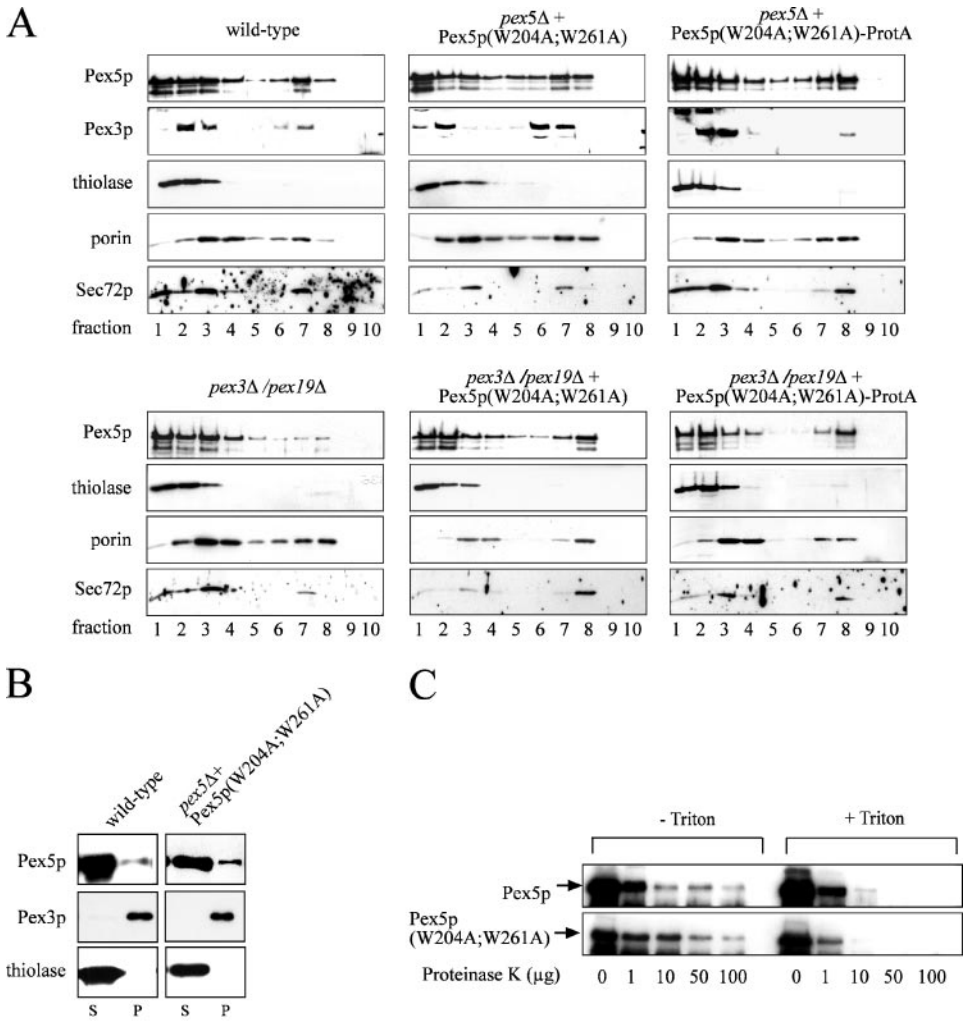

FIGURE 4. **Pex5p(W261A;W204A) and wild-type Pex5p exhibit the same membrane properties.** *A*, membrane association of Pex5p is not affected by the point mutations W204A and W261A. Oleate grown wild-type cells, *pex3*Δ*pex19*Δ cells, and *pex5*Δ cells expressing Pex5p-ProtA, Pex5p(W204A)-ProtA, Pex5p(W261A)-ProtA, or Pex5p(W204A;W261A)-ProtA under control of the endogenous promotor were disrupted with glass beads. 1.5 mg of protein from the lysate was applied to a flotation gradient. After ultracentrifugation at 170,000 × *g* the gradient was collected as 10 fractions from top (fraction 10) to bottom (fraction 1). Equal portions of each fraction were subjected to immunoblot analysis with antisera directed against Pex5p, Pex3p, thiolase, Sec72p, and porin. *B* and *C*, organellar fractions were obtained from lysates of wild-type and *pex5*Δ cells expressing Pex5p(W204A;W261A) by a 25,000 × *g* centrifugation step and applied to alkaline and proteinase K treatment. The organelles were incubated with 0.1 M sodium carbonate, pH 11.5, for 30 min (*B*). Extracted proteins (*S*, supernatant) were separated from the membrane fraction (*P*, pellet) by centrifugation. Equal amounts of proteins were analyzed by Western blotting for the presence of Pex5p, the integral membrane protein Pex3p, and the matrix protein thiolase. The membrane topology of wild-type Pex5p and mutant Pex5p(W204A; W261A) was tested by treating organellar pellets for 10 min with varying amounts of proteinase K and analyzed by Western blotting using anti Pex5p antibodies (*C*).

mutants as described previously (30). To achieve insertion of Pex5p into membranes first, cytosolic fractions from Pex1p-deficient cells containing wild-type or mutant Pex5 proteins were incubated with whole cell membranes obtained from *pex1*Δ*pex5*Δ cells. Like wild type, significant amounts of the single and double mutants associate with the membranes *in vitro* (Fig. 5*A*, import pellet fractions). Further sample analyses revealed that all membrane-associated Pex5 proteins can be released again (Fig. 5*A*, export supernatant fractions). The export of wild-type and mutant proteins depends on the presence of ATP and Pex1p, demonstrating that all tested variants are substrates for AAA peroxins and ATP-dependent export and thus are capable of cycling between the peroxisomal and cytosolic compartment. The fact that only peroxisomal membranes contain functional Pex5p export machinery further proves that a considerable amount of Pex5p(W204A;W261A) enters the right compartment. However, the relative amounts of Pex5p mutants in the release fractions are lower when compared with wild type. In particular, Pex5p(W204A) and Pex5p(W204A;W261A) are drastically reduced. One possible explanation could be that the interactions between the mutated PTS1 receptors and the docking complex, in particular Pex13p, are critical for an efficient transport of Pex5p along and from the peroxisomal membrane. This notion is further supported by using Pex5p-associated membrane preparations from *pex13*Δ*pex14*Δ cells in the *in vitro* export assay (Fig. 5*B*). In the absence of both Pex13p and Pex14p, no specific release of membrane-bound Pex5p could be detected.

Human and Yeast Pex5p Can Insert Spontaneously into Phospholipid Membranes in Vitro—The results described above indicate that yeast Pex5p might not require stable interactions with any of the tested membrane-bound peroxins for its functional association with the peroxisomal membrane. In order to study the nature of the ability of Pex5p to bind to peroxisomal membranes, we investigated the potential of Pex5p from human and yeast to interact directly with lipids. As a first approach, we tested the insertion of recombinant purified PTS1 receptors into phospholipid monolayers. All experiments were carried out with a phospholipids mixture consisting of 3 volumes DOPE and 7 volumes DOPC. This composition was based on previous reports demonstrating that these lipids are the principal constituents, 28% phosphoethanolamine and 57% phosphocholine, respectively, of the membranes of rat liver peroxisomes (54). A mixture of both was spread on an aqueous subphase to form monolayers at the air/water interphase with an initial surface pressure of 20 mN/m. The insertion of *Sc*Pex5p and *Hs*Pex5p purified from *Escherichia coli* extracts was monitored after their injection below the monolayer surface. In both experiments, monolayer surface pressure

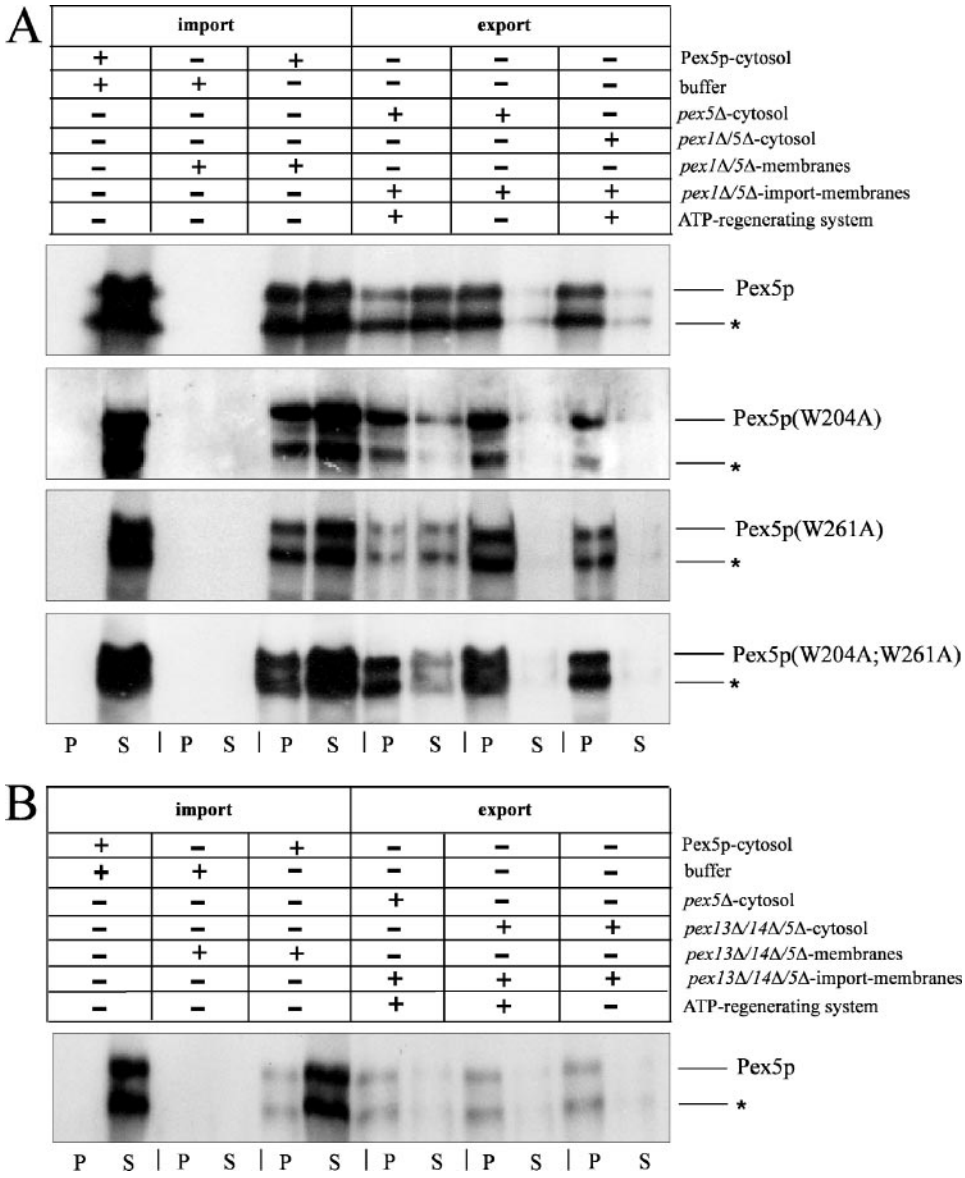

FIGURE 5. **Pex5p(W204A;W261A) can cycle between the peroxisomal membrane and the cytosol *in vitro*.** *A*, Pex5p(W204A), Pex5p(W261A), and Pex5p(W204A;W261A) can be exported from peroxisomal membranes in the presence of ATP and the AAA-ATPase Pex1p. The *in vitro* assays were based on cytosol- and membrane-fractions derived from 125 mg of oleate-grown cells. For import reaction, *pex1*Δ*pex5*Δ cytosols complemented with different plasmid-encoded Pex5p forms (Pex5p-cytosol) as indicated were incubated with *pex1*Δ*pex5*Δ membranes for 20 min at 26 °C in four identical samples. After separation of membrane and supernatant fractions via a 100,000 × *g* centrifugation step, the fractions of one sample were subjected to trichloroacetic acid precipitation. For export reaction, the pellet fractions of the other three identical samples, containing the Pex5p-import-membranes, were solubilized in either *pex5*Δ cytosol (containing an ATP-regenerating system), *pex5*Δ cytosol (without an ATP-regenerating system), or *pex1*Δ*pex5*Δ cytosol (with an ATP-regenerating system). Samples were incubated for 20 min at 37 °C and separated again via centrifugation. The isolated fractions were subjected to trichloroacetic acid precipitation. *Asterisks* mark degradation products of Pex5 proteins. *B*, the presence of both Pex14p and Pex13p is essential for *in vitro* recycling of Pex5p back to the cytosol. The *in vitro* import and export reactions were performed as described above, with the difference that *pex5*Δ cytosol complemented with plasmid-encoded Pex5p as well as *pex13*Δ*pex14*Δ*pex5*Δ membranes were used.

increased as a function of the amount of Pex5p injected resulting in maximal increases of 5 and 7 mN/m, respectively (Fig. 6*A*). In the absence of a lipid film, the proteins themselves showed a surface pressure increase less than 1 mN/m (data not shown). Thus, we conclude that the increase in surface pressure was not due to the surface properties of the proteins but to the insertion of Pex5 proteins into the lipid monolayer. The specificity of this lipid binding assay is demonstrated by the usage of two other purified soluble proteins, both of which play major roles in bacterial protein translocation processes. SecA, one of the two control proteins, has been reported to possess lipid binding activity as shown previously by monolayer experiments (55). Using our assay conditions, SecA affects the surface pressure in a similar range as the PTS1 receptors, whereas the cytoplasmic receptor SecB remained in the water phase without a significant disturbance of the surface tension (Fig. 6*B*) (56).

To extend the studies concerning the lipid binding activity of the recombinant PTS1 receptors for bilayer membranes, we incubated human and yeast Pex5p with artificial liposomes generated by a phospholipid mixture DOPE:DOPC (3:7). Subsequently, the vesicles were floated by sucrose density gradient centrifugation, and the fractions were analyzed by Western blotting. Fig. 5*B* shows that a significant portion of both *Hs*Pex5p and *Sc*Pex5p comigrate with the floated liposomes. Although in both preparations many degradation products of Pex5p could be detected, only the full-length and in the case of *Sc*Pex5p one additional degradation product are found in the lighter fractions of the gradients. The fact that some major degradation products do not float with liposomes demonstrates that the liposome association of the intact PTS1 receptors is not due to unspecific binding. To address the question if the full-length Pex5 proteins associate peripherally with the liposomes or insert into the bilayer phase, we further applied protease protection assays to Pex5p fractions (Fig. 5*C*). A significant fraction of both human and yeast Pex5p became resistant to trypsin treatment when associated with liposomes. This result is in line with previous studies demonstrating that human membrane-bound Pex5p remains uncleaved when intact organelles were incubated with trypsin (15). Taken together, our *in vitro* studies suggest that both PTS1 receptors can insert spontaneously into bilayer membranes.

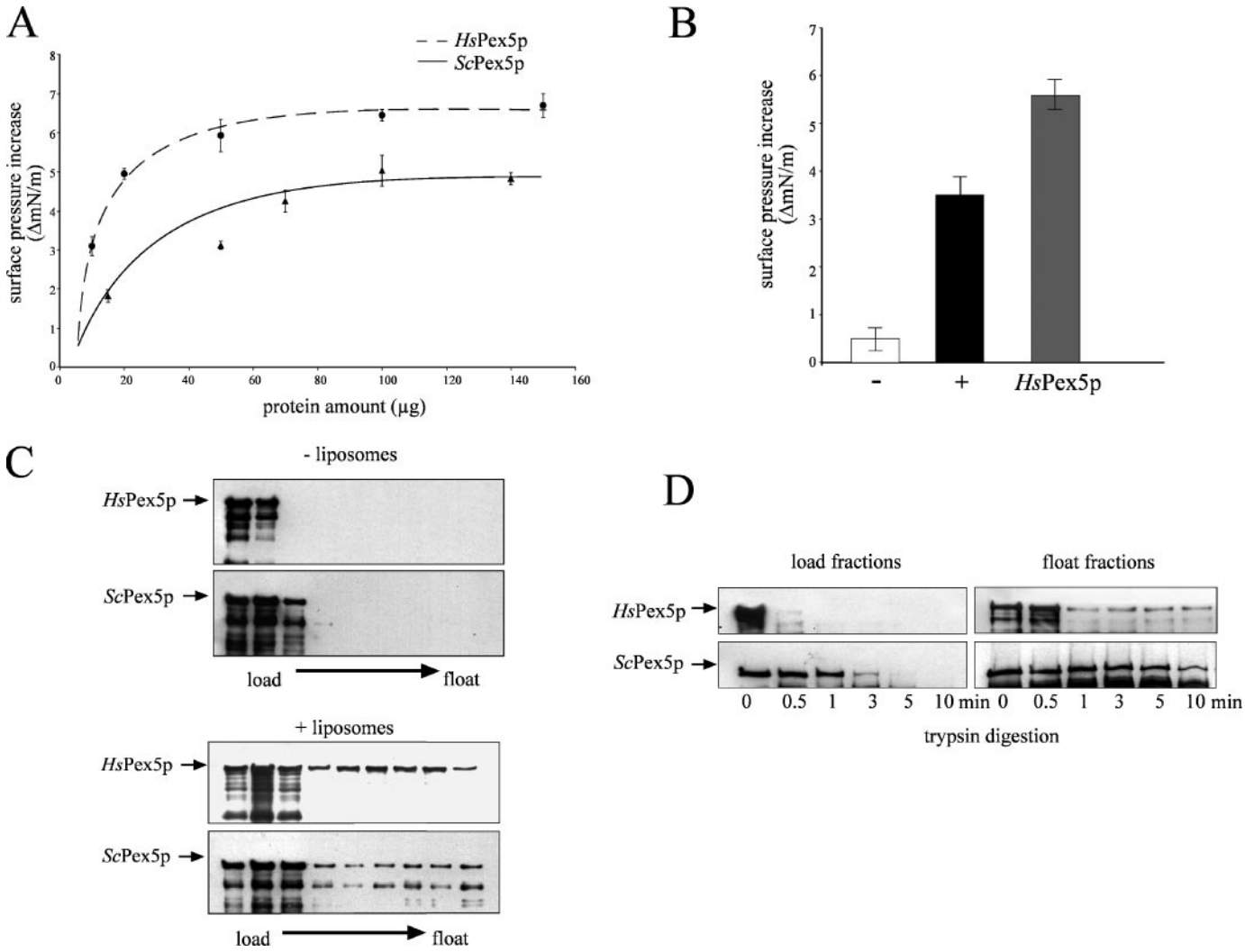

FIGURE 6. **Spontaneous *in vitro* insertion of human and yeast Pex5p into phospholipid membranes.** *A*, both human and *S. cerevisiae* Pex5p can insert into a phospholipid monolayer. The increase of monolayer surface pressure was measured as a function of the amount of Pex5p injected into the subphase below a monolayer composed of 30% DOPE and 70% DOPC. The initial surface pressure was adjusted to 20 mN/m. *Dashed line, Hs*Pex5p; *solid line, Sc*Pex5p. *B*, to verify the obtained *in vitro* insertions, the cytoplasmic protein SecB (*white bar*; negative control) and the membrane protein SecA (*black bar*; positive control) were used as controls. Same amounts (100 μg) of these proteins and human Pex5p (*gray bar*) were injected below a monolayer (30% DOPC, 70% DOPE; initial surface pressure 20 mN/m). Standard errors are indicated. *C*, recombinant Pex5p associates with phospholipid bilayer vesicles. Purified human and *S. cerevisiae* Pex5p (0.75 nmol) were incubated for 1 h at room temperature with and without liposomes (ratio 1:750). The samples were subjected to flotation gradient centrifugation. The gradient was collected as 10 fractions from top to bottom. Equal volumes of fractions were analyzed using antibodies against human or yeast Pex5p. *D*, liposome-associated Pex5p is partly protease-resistant. Load and float gradient fractions containing human or yeast Pex5p were mixed with trypsin in a 60:1 ratio. After digestion for 0, 0.5, 1, 3, 5, and 10 min, samples were taken and analyzed by Western blotting.

DISCUSSION

During each receptor cycle the cytosolic PTS1 receptor Pex5p binds tightly to the peroxisomal membrane. It is generally believed that cargo-laden receptor associates with the membrane via protein-protein interactions. This view is primarily based on the lack of predictable membrane spans in the sequence of Pex5p and its direct binding to several membrane-bound peroxins, including the docking complex constituents Pex14p and Pex13p as well as the RING finger peroxin Pex12p (2). Here we demonstrate that Pex5p has the ability to insert in an import-competent state into the peroxisomal membrane without a stable association with these peroxins. This conclusion is based on the following lines of *in vivo* and *in vitro* evidence. First, lipid binding activity of both human and yeast Pex5p could be demonstrated directly by spontaneous insertion of the purified proteins into artificial phospholipid mono- and bilayers. Second, in a PEX5-deleted yeast strain we have expressed a mutant, Pex5p(W204A;W261A), defective in the interaction with Pex13p and Pex14p. These cells contain cargo-laden PTS1 receptor in the peroxisomal membrane and are able to grow on oleate as sole carbon source. This mutated *Sc*Pex5p variant still facilitates the import of PTS1 proteins and can be exported from the membrane in a Pex1p- and ATP-dependent manner. However, the active mutant has lost the ability to form a stable complex with any of the components of the docking complex and in addition with Pex10p and Pex12p suggesting that it is held by protein-lipid interactions rather than protein-protein interactions in the peroxisomal membrane.

For disconnecting the PTS1 receptor from the docking and RING finger complexes, we have identified the binding site of yeast Pex5p for the conserved N-terminal domain of Pex14p. This region was shown to interact directly with W*XXX*F motifs of mammalian and plant Pex5p. Although yeast Pex5p contains two of these motifs, these are not directly involved in Pex14p binding. Remarkably, the mapped Pex14p binding region contains a single reverse W*XXX*F motif that is conserved among fungi and *C. elegans*. Site-directed mutagenesis of the conserved tryptophan into an alanine (W261A) within this novel motif confirmed the importance of this residue for the Pex14p-N binding and thereby emphasized the similarity between W*XXX*F motifs (in which tryptophan is a key residue) and the F*XXX*W motif in the mode of binding to the N-terminal domain of Pex14p.

As Azevedo and co-workers (57) reported that the insertion of mammalian Pex5p into the peroxisomal membrane requires Pex14p, we expected that this W261A mutation in yeast Pex5p would lead to an exclusively soluble PTS1 receptor. However, our data show that the cargo-laden PTS1 receptor still associates with the docking and the RING finger complex most likely via its interaction with Pex13p (see Fig. 2*C*). Therefore, we combined the mutation within the reverse W*XXX*F motif, W261A, with a single point mutation within the Pex13p-binding site W204A. In fact, introduction of both mutations into Pex5p abolished the formation of stable Pex5p-docking-RING finger complexes.

Surprisingly, the mutations did not prevent the membrane association of the PTS1 receptor (Fig. 2*A* and Fig. 4*A*), whereas subcellular fractionation analyses indicate that the double mutant Pex5p is localized not only to peroxisomal but also to other cellular membranes (Fig. 3*D*). The partial mislocalization of the double mutant to other non-peroxisomal organelles/membranes suggests that the formation of a stable Pex5p-docking complex contributes to the specific association of Pex5p with the peroxisomal membrane. Accordingly, the amount of peroxisome-associated Pex5p seems to be negligible in *pex13*Δ*pex14*Δ cells (Fig. 5*B*). With respect to the partial peroxisomal association of Pex5p(W204A,W261A), it is relevant to note that *Sc*Pex14p possesses a second binding site for Pex5p (41, 49). Although the first Pex5p-binding site of *Sc*Pex14p is within the membrane protected N-terminal domain (58), the second one is located at the cytosolic C-terminal end of Pex14p and is reported to provide the docking function (41). It is important to note that the presented results also indicate that Pex5p possesses two independent binding sites for Pex14p as follows: the inverted W*XXX*F motif for the Pex14p-N representing the conserved binding mode, and another one for Pex14p-C that has not yet been mapped and that so far has been found only in *S. cerevisiae*. It may well be possible that this second binding site transiently interacts with Pex5p. This could also explain the residual less than 2% of Pex14p found associated with Pex5p(W204A;W261A).

A second totally unexpected property of Pex5p harboring the two point mutations, W204A and W261A, is that it still mediates the import of peroxisomal matrix proteins which in turn allows cells expressing this mutant Pex5p to grow on oleate. These results indicate that the formation of a stable complex between Pex5p and constituents of the docking and the RING finger complex is not a stringent requirement to retain PTS1 import activity.

How can these surprising results be reconciled with the fact that Pex14p- or Pex13p-deficient cells are completely blocked in matrix protein import (23, 24, 42). The answer could be very simple. AlthoughPex5p(W204A;W261A) definitely has lower affinity for proteins in the docking complex, the affinity *in situ* may still be sufficient to permit partial Pex5p function along the normal pathway. This assumption is supported by the finding that the double mutant Pex5p can be released by Pex1p, an event definitely acting downstream of the formation of a docking complex. Although this concept of transient protein-protein interactions could account for partial functionality of PTS1 import, it is more difficult to understand that Pex5p(W204A;W261A) exhibits the same membrane binding properties as wild-type Pex5p without being stably associated with its predominant protein binding partners Pex14p and Pex13p. Therefore, we considered the possibility that Pex5p itself has the property to bind directly to lipids. The observed *in vitro* insertion of Pex5p into phospholipid mono- and bilayer strongly supports this notion and could explain the vesicular localization of Pex5p(W204A;W261A) in a *pex3*Δ*pex19*Δ strain lacking peroxisomal membranes (Fig. 4*A*).

The newly discovered intrinsic lipid-binding properties of Pex5p together with the results obtained with an active Pex5p mutant that is no longer stably associated with its known binding partners open the possibility that a lipid-bound intermediate of the receptor-cargo complex might exist under physiological conditions. The complex network of interactions with its initial binding partners suggests a novel sequence of events for the docking step. The most simple interpretation of all of these findings regarding the properties of Pex5p and Pex14p would be that the C-terminal binding site of Pex14p tethers the Pex5p-cargo complexes to the membrane and thereby triggers its insertion into the membrane. Subsequently, the N-terminal binding site of Pex14p together with Pex13p stably connects the membrane-embedded Pex5p with the docking and the RING finger complex. In this model the N-terminal domain of Pex14p is acting like an adaptor domain, which could help to increase the efficiency of matrix protein translocation and/or receptor recycling.

There is additional experimental evidence that supports this view. Salomons *et al.* (59) reported that in the yeast *Hansenula polymorpha* a massive overproduction of Pex5p can partially rescue the import defect of Pex14p-depleted cells. This could mean that a high concentration of Pex5p partially compensates for the lacking tethering step, and under these circumstances Pex5p inserts without any help into the membrane.

The proposed but still not yet proven model of the initial events at the peroxisomal membrane can be experimentally tested, *e.g.* by identification of the lipid-binding sites of Pex5p and generation of mutants that fail to associate with liposomes *in vitro* and peroxisomes *in vivo*. Such experiments to evaluate the physiological relevance of a lipid-bound state of Pex5p are underway.

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2.4

Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling.

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(zur Publikation eingereicht)

Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling

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Pex5p, the import receptor for peroxisomal matrix proteins harbouring a type I signal sequence (PTS1), is mono- and polyubiquitinated in *Saccharomyces cerevisiae*. We demonstrate that monoubiquitination of Pex5p depends on Pex4p (Ubc10p) and that either poly- or monoubiquitination is required for the ATP-dependent release of Pex5p from the peroxisomal membrane to the cytosol as part of the receptor cycle.

The peroxisomal import receptor Pex5p binds its cargo proteins in the cytosol and targets them to a docking and translocation machinery at the peroxisomal membrane (reviewed in[1]) where the receptor releases the cargo proteins into the peroxisomal lumen and shuttles back to the cytosol. Recently, we discovered that the dislocation of the yeast PTS receptor Pex5p from the peroxisomal membrane to the cytosol after cargo release is performed by the peroxisomal AAA-proteins Pex1p and Pex6p. This was shown to be also true for the mammalian system[2, 3]. Moreover, we and others showed that Pex5p is modified by mono- and polyubiquitination[4-6], but the functional role of this modification was not known.

The study of the role of the monoubiquitination of Pex5p observed in wild-type cells [6] has been hampered by the polyubiquitination of Pex5p, which accumulates at the peroxisomal membrane in cells lacking components required for the late steps in the import pathway [4, 5]. In order to study Pex5p monoubiquitination, we followed two different strategies to avoid polyubiquitination. For both strategies, we assumed the polyubiquitination site to reside within the N-terminal region of the protein, since this region of Pex5p from human and rat is sufficient to carry out its docking to the peroxisomal membrane [7, 8] as well as the consecutive dislocation from the membrane to the cytosol [8]. On the one hand, we fused three *myc*-epitopes to the amino-terminus of Pex5p, which for other proteins has been demonstrated to prevent polyubiquitination [9]. On the other hand, we substituted the first conserved lysine residue (lysine 18) of Pex5p by site directed mutagenesis. The corresponding lysine residue has been demonstrated to be required for polyubiquitination of Pex5p from *Hansenula polymorpha* [10].

However, in our case, the single mutant protein (Pex5pK18R) was still polyubiquitinated (data not shown). Thus, we considered that an adjacent lysine might substitute for the loss as it is the case for many other proteins destined for degradation [11] and we accordingly additionally replaced lysine 24 of Pex5p by arginine. Both the *myc*-tagged Pex5p as well as Pex5pK18/24R fully restored the growth defect of a *PEX5*-deletion strain on medium with oleate as sole carbon source (data not shown) and imported GFP-PTS1 properly into peroxisomes **(Fig. 1A)**. Moreover, *myc*Pex5p as well as Pex5pK18/24R were normally bound and released from the peroxisomal membrane in an *in vitro* system **(Fig. 1B).** Thus, both variants behaved like the wild-type protein, thereby demonstrating that neither the tag nor the introduced mutations interfered with the physiological role of Pex5p in peroxisomal protein import. Significantly, when transformed into *pex4*Δ*pex5*Δ or *pex1*Δ*pex5*Δ strains, no polyubiquitinated forms of the *myc*-tagged or mutated Pex5p could be detected in the cell lysates **(Fig. 1C).** The same was true for the temperature sensitive proteasomal mutant strain *cim5-1pex5*Δ (data not shown). Thus, the exchange of the lysine residues deleted the target residues for ubiquitination. Similarily, the N-terminal *myc*-tagging prevented the polyubiquitination, possibly by interfering with polyubiquitin specific factors such as Ubc4p/Ubc5p or the corresponding E3-enzyme, or by masking the target lysine residues for polyubiquitin chain formation. The fact that *pex5Δ*-cells harbouring *myc*Pex5p or Pex5pK18/24R did not exhibit a growth defect on oleic acid medium and import GFP-PTS1 indicates that polyubiquitination is not prerequisite for functional peroxisomal protein import in *Saccharomyces cerevisiae*. These data are in agreement with the idea that polyubiquitination is part of a quality control system that primes membrane-accumulated Pex5p for proteasomal degradation [3, 5, 12].

Despite the lack of polyubiquitination, *myc*Pex5p and Pex5pK18/24R were still normally monoubiquitinated**.** Preparation of membrane pellets in the presence of N-ethylmaleimide (NEM) in order to inhibit deubiquitinating enzymes results in the appearance of a more

slowly migrating form of Pex5p which has been demonstrated to represent mono-ubiquitinated Pex5p [6]. This is also observed for *myc*Pex5p or Pex5pK18/24R **(Fig. 1D)**. The more slowly migrating form did shift to a higher molecular weight upon expressing of *myc*Ub, demonstrating its ubiquitin nature. No change in molecular weight was observed when mutated ubiquitin (UbK48R) was expressed, which prevents the formation of polyubiquitin chains [13], confirming the mono-ubiquitination of the proteins. Thus, with respect to monoubiquitination, the myc-tagged and the K18R/K24R-double mutated Pex5p behave like the endogenous protein.

It has been reported that monoubiquitination of Pex5p is independent of *UBC8*, *UBC5*, *UBC4*, *UBC1* and even takes place in a *UBC1/UBC4*-double deletion strain [6]. However, the observations on monoubiquitination were hampered in mutants lacking UBC10 (Pex4p) and other late peroxins like Pex1p and Pex6p because of the presence of polyubiquitinated Pex5p. Here, we could take advantage of the fact that *myc*Pex5p is not polyubiquitinated but is still susceptible to monoubiquitination, enabling us to investigate which of the known UBCs is responsible for the monoubiquitination event. We isolated *myc*Pex5p via immunoprecipitation from chosen *UBC*-deletion strains, leaving out the lethal deletion of *UBC3* as well as of the SUMO-conjugating enzyme Ubc9p and the Nedd8/Rub1-conjugating enzyme Ubc12p. In all the UBC-deletion strains tested (*ubc1Δ*, *ubc2Δ*, *ubc4Δubc5Δ*, *ubc6Δ*, *ubc7Δ, ubc8Δ*, *ubc11Δ*, *ubc13Δ*), formation of mono-Ub-*myc*Pex5p was visible, except in the case of Pex4p (Ubc10p) deletion, which was completely prevented in the conjugation of ubiquitin molecules to *myc*Pex5p **(Fig. 2A).** These data clearly demonstrate the dependence of *myc*Pex5p monoubiquitination on the presence of the ubiquitin-conjugating enzyme Pex4p. In order to determine whether not only the presence but also the catalytic activity of Pex4p is essential for Pex5p monoubiquitination, we expressed an inactive Pex4p-mutant protein, which carries a C to S point mutation at position 115. This aminoacid residue is essential for the activity of ubiquitin-conjugating enzymes [14]. Although Pex4p(C115S) can be expressed to nearly wild-

type levels and is properly targeted to peroxisomes [15], monoubiquitination of the *myc*-tagged or point-mutated PTS1-receptor was completely abolished **(Fig. 2B; suppl. Fig. 1A).**

In addition to its catalytic activity, the peroxisomal localization of Pex4p also proved to be essential for Pex5p-monoubiquitination. *myc*Pex5p or Pex5pK18/24R was expressed in a *pex22*Δ background, which lacks the peroxisomal membrane anchor for Pex4p [16]. As shown in **Fig.2B,** monoubiquitination of Pex5p was not observed in the *pex22*Δ strain**.** The fact that the presence of an active ubiquitin-conjugating enzyme Pex4p attached to the peroxisomal membrane via Pex22p is indispensable for the formation of monoubiquitinated Pex5p indicates that the PTS1-receptor is a physiological substrate of Pex4p. The question is whether this is final proof that Pex5p is the long searched for molecular target of Pex4p-dependent ubiquitination. It could still be argued that our findings might be explained by a function of Pex4p in the assembly of a so far undefined complex essential for Pex5p-ubiquitination or involvement of Pex4p in the transfer of Pex5p to the place of its monoubiquitination. Thus, the ultimate answer still awaits the demonstration of the Pex4p-function in an *in vitro* system. However, several lines of evidence strongly support the idea of Pex4p playing a direct role in Pex5p-ubiquitination. First, Pex4p is an UBC that has already been demonstrated to bind ubiquitin and based on our data the dependence of Pex5p-monoubiquitination on Pex4p also is unquestionable. Second, as already described, site directed mutagenesis of Pex4p substituting a conserved cysteine residue (cysteine 115), which was demonstrated to be essential in all known UBCs [14] does not interfere with Pex4p localization [15] but prevents Pex5p-monoubiquitination. Finally, one of the known eleven UBCs in yeast [14] must be involved in Pex5p-monoubiquitination. Thus, the question is, if not Pex4p, which UBC could be responsible for Pex5p-ubiquitination. Of the nine non-essential UBCs, only Pex4p proved to be required for Pex5p-monoubiquitination. Thus, the collected data indicate that Pex5p is the molecular target for monoubiquitination by Pex4p.

Next we addressed the question whether Pex4p is required up- or downstream of the AAA-complex that is responsible for Pex5p release from the peroxisomal membrane [2, 3]. Previous findings demonstrated that the receptor docking at the peroxisomal membrane and transfer to the RING-finger peroxins is prerequisite for monoubiquitination [6]. Another attempt to elucidate the order of events was made by Collins and co-workers in *P. pastoris* [17]. They took advantage of a specific instability of Pex5p in mutant strains lacking components of the AAA- and Pex4p/Pex22p-complex. Based on the finding that the Pex5p-level in a *pex1*Δ/*pex4*Δ strain was reduced to the level of the *pex1*Δ single mutant strain, it was concluded that Pex4p acts downstream of the AAA-peroxins [17]. In *Saccharomyces cerevisiae pex1*Δ-cells, such a Pex5p-instability is not observed but the protein becomes polyubiquitinated and accumulates at the peroxisomal membrane. Thus, the observed Pex5p instability in other yeasts is likely to be a consequence of polyubiquitination and subsequent proteasomal degradation. In this case, the Pex5p polyubiquitination seems to be part of a quality control system that is not directly related to the import process. Instead of this pathological situation, we now took advantage of the physiological monoubiquitination that is also present in wild-type cells to study the epistasis. The analysis revealed that both *myc*Pex5p and Pex5pK18/24R, which are monoubiquitinated under wild-type conditions but not in *PEX4* affected cells, are still monoubiquitinated in a *pex1*Δ strain. In contrast, ubiquitination did not take place in a *pex4Δpex1Δ* double deletion strain **(Fig. 2C; Suppl. Fig. 1B)**. This result demonstrates that Pex4p-dependent monoubiquitination occurs independently of the presence of the AAA peroxins and thus prior to the function of the Pex1p/Pex6p-complex in the peroxisomal protein import pathway. Thus, monoubiquitination of Pex5p takes place before the protein is released from the peroxisomal membrane in an AAA-peroxin and ATP-dependent manner. An explanation for the different conclusion drawn by Collins and co-workers is provided by the different nature of the Pex5p-fraction analyzed. Collins and co-workers used the instability of Pex5p as an indicator and thus most probably analyzed the Pex5p form

designated for proteasomal degradation [17], a process for which Pex4p has been demonstrated to be dispensable [4]. Monoubiquitination requires Pex4p and thus is likely to represent an important step in the peroxisomal protein import process. Thus, the epistasis on the basis of monoubiquitination is expected to reflect the sequence of events in the Pex5p receptor cycle.

We then asked whether ubiquitination of Pex5p is a prerequisite for the release of Pex5p from the peroxisomal membrane to the cytosol. Previously, we demonstrated that Pex5p can only be exported from membranes derived from a *pex1*Δ strain when incubated with cytosol containing the AAA-peroxins, or with the isolated AAA complex [3]. To test for the ubiquitin-requirement, we aimed to delete all possible ubiquitination sites of Pex5p. For the prevention of polyubiquitination this was achieved by deletion of K18/24R. However, deletion of any of the 15 lysines within the N-terminal half of Pex5p did not abolish the monoubiquitination of the receptor, indicating that the absence of one ubiquitination site could be overcome by using another one. Therefore, we took advantage of the fact that Pex5p is not monoubiquitinated in *pex4Δ*-cells. The lack of Pex5p-monoubiquitination in *pex4Δ* and the prevention of polyubiquitination of Pex5pK18/24R enabled us to separately investigate the contribution mono- and polyubiquitination to Pex5p release from the peroxisomal membrane. **Fig.3** shows that Pex5pK18/24R is still exported from the peroxisomal membrane in an AAA-peroxin and ATP-dependent manner. Similarily, in a Pex4p-deficient system reflected by *pex4*Δ membranes incubated with a *pex4Δ/pex5*Δ cytosol, a fraction of endogenous encoded Pex5p was released from the membrane. This liberation of Pex5p from cells lacking Pex4p still required the presence of ATP and the activity of the AAA peroxins. However, when Pex5pK18/24R was subjected to the export assay in a Pex4p-deficient system, release of the receptor from the membrane was completely blocked. Thus, the simultaneous loss of both polyubiquitination and monoubiquitination of the receptor prevented release of the receptor, demonstrating that Pex5p-ubiquitination is required for its release from the membrane.

Our findings also show that mono- or polyubiquitination are both sufficient to prepare Pex5p for the AAA-dependent release to the cytosol. In the case of p53 as well as Rad18, it was reported that their monoubiquitination is a prerequisite for export from the nucleus. In contrast, polyubiquitination of these proteins leads to proteasomal degradation [18, 19]. In analogy, the fate of the released mono- or polyubiquitinated Pex5p is proposed to be different as outlined in the model depicted in **Fig. 4**. After its release, the polyubiquitinated Pex5p is directed to proteasomal degradation as part of a quality control system that withdraws Pex5p from the import cycle. In support of this assumption, prevention of polyubiquitination by either deleting the corresponding UBCs Ubc4p and Ubc5p [4, 5] or substitution of the target lysine residues evidently did not interfere with Pex5p function nor with its AAA-peroxin dependent release from the peroxisomal membrane. In contrast, according to our model, the released monoubiquitinated Pex5p is supposed to be deubiquitinated and made available for further rounds of matrix protein import. This provides a plausible explanation for the PTS1-import defect of a *PEX4* deletion strain and for the previously observed accumulation of Pex5p in cells lacking Pex4p [4, 20]. As *pex4*Δ cells also exhibit a PTS2-import defect, one could assume a similar role of Pex4p in the cycle of the PTS2-receptor or the auxiliary proteins Pex18p/Pex21p.

We have demonstrated here that ubiquitination of the Pex5p is a prerequisite for its dislocation from the peroxisomal membrane by the AAA-peroxins. Ubiquitination of Pex5p is expected to facilitate the recruitment of the AAA-machinery. The functional role of Pex4p/Ubc10p, which is the only ubiquitin-conjugating enzyme known to be involved in the biogenesis of an organelle, has been a mystery for nearly 13 years. Here we provide conclusive evidence for Pex5p being the molecular target for monoubiquitination by Pex4p and show a direct role for the protein in the membrane release of Pex5p at the end of the import cascade. It has been demonstrated that the ATP-consuming step in this process is not the binding and import of Pex5p but the AAA-peroxin dependent export of the receptor [2, 21].

As monoubiquitination is essential for efficient receptor recycling, we have expanded the energy-requirement of the peroxisomal import pathway by a second ATP-dependent step, namely receptor-monoubiquitination.

METHODS

Yeast strains and culture conditions

The *Saccharomyces cerevisiae* strain UTL-7A (MATa, ura3-52, trp1, leu2-3/112) [22] was used as an isogenic wild-type strain for the generation of wild-type, *pex1*Δ [4], *pex1Δ/4*Δ [4], *pex1Δ/5Δ* [3], *pex4Δ* [15], *pex4Δ/5Δ* (this study), *pex4Δ/5Δ/1Δ* (this study), *pex5Δ* [23], *pex22*Δ5Δ (this study), as well as *ubc1Δ*, *ubc2Δ*, *ubc4Δ/ubc5Δ*, *ubc6Δ*, *ubc7Δ*, *ubc8Δ*, *ubc11Δ* and *ubc13Δ* strains [4]. Deletion strains were generated by the `short flanking homology` method as described before [24]. Strains in which the genomic copies of genes express proteins fused to TEV-ProtA were produced according to [25]. Yeast media have been described previously [22].

Plasmids and cloning strategies

The PEX5-ORF was amplified using the oligonucleotides RE1168 (5`-GTCAGTGTCGACCATGGACGTAGGAAGTTGCTCA-3`) and RE1169 (5`-GCAGAGATCTTCAAAACGAAAATTCTCCTTTAAATC-3`). The template plasmid pWK19 contains the wild-type *ScPEX5*, which previously had been amplified from genomic DNA of the UTL-7A wild-type strain. The PCR product was digested with *Sal*I and *Bgl*II and ligated into the pWK13-vector, a *PEX5*-promotor containing low copy-vector, resulting in pHP17-PEX5. Plasmids pWK19 and pWK13 were kindly provided by Wolfgang Schliebs (Bochum, Germany).

Point mutations in *PEX5* were introduced using overlap extension PCR. For the K18R exchange, PCR-products were amplified by primer pairs RE1168/RE1149 and RE1169/RE1150, resulting in substitution of the lysine codon sequence AAA to the arginine codon sequence AGA. RE1149 (5`-CCGCTTGCGCAGTTGCACAGACATACTCAGCAGAACAAA-3`) and RE1150 (5`-TTTGTTCTGCTGAGTATGTCTGTGCAACTGCGCAAGCGG-3`) contain a substituted base pair triplett coding for arginine at amino acid position 18. The external primers RE1168 and RE1169 together with both PCR-products were used for the overlap extension PCR. The amplification product was digested with *Sal*I and *Bgl*II and ligated into *Sal*I/*Bgl*II digested pWK13, resulting in pHP1-*PEX5* (K18R). To introduce the double mutation K18R/K24R into the *PEX5* sequence, the construct pHP1-*PEX5* (K18R) was used as a template. For substitution of the lysine triplett AAA to the arginine triplett AAC at amino acid postion 24, overlapping PCR was carried out as described above using the external primers RE1168 and RE1169 as well as oligonucleotides RE1285 (5`-AAACATACTCAGCAGAACAGATCGCTTCAGTTTAATCAG-3`) and RE1286 (5`-CTGATTAAACTGAAGCGATCTGTTCTGCTGAGTATGTTT-3`). The PCR product was digested with *Sal*I and *Bgl*II and ligated into *Sal*I and *Bgl*II digested pWK13-vector, resulting in pHP18-PEX5 (K18R;K24R).

Plasmids expressing *myc*Pex5p (pRS6*myc*$_3$) (this study), Pex4p^{C115S} (pEMBL*yex*-PAS2^{Ser})[15], PTS1-GFP [26] and Ubiquitin (YEp96)[27], Ubiquitin (K48R)(YEp110) [28] as well as *myc*Ubiquitin (YEp105) [29] were used.

Membrane Sedimentation

Oleic-acid induced cells were washed with H_2O and 1g was used per sedimentation. Three milliliters of buffer A (0.2 M HEPES, 1 M KOAc, 50 mM MgOAc, pH 7.5), protease inhibitors (8 µM antipain, 0.3 µM aprotinin, 1µM bestatin, 10 µM chymostatin, 5 µM leupeptin, 1.5 µM pepstatin, 1mM benzamidin, 1 mM PMSF (Boehringer Mannheim)), 5 mM NaF and 3g glass beads (0.5 mm) were added to the cells. For the preparation of mono-ubiquitinated Pex5p, additionally 20 µM NEM (Sigma) were added to the lysis buffer. Breakage was achieved by vortexing for 12 min (12 x 60 s with breaks of at least 60s on ice). Samples were transferred to Corex tubes and centrifugated at 1,500 x g for 10 min. Supernatants were normalized for protein and volume and membranes were sedimented at 100,000 x g for 30 min (Sorvall AH650, 40850 rpm). The resulting pellet was resuspended in buffer A corresponding to the volume of the supernatant. Aliquots of the samples were analyzed by SDS-PAGE.

Miscellaneous

In vitro import and export assays were performed according to [3]. Protein complexes were isolated by co-immunoprecipitation as described by [23]. TCA-lysates of cellular fractions were prepared as described by [4]. Live cell imaging for GFP fluorescence was performed with a Zeiss Axioplan microscope and AxioVision 4.1 software (Zeiss, Jena, Germany). Immuno-reactive complexes were visualized using anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase in combination with the ECL™ system from Amersham Pharmacia Biotech (Uppsala, Sweden). Polyclonal rabbit antibodies were raised against Pex5p [30], Pex13p [23] and Fructose-1,6-bisphosphatase [31]. Monoclonal mouse antibodies were raised against the C-*myc* epitope [32].

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Figure Legends

Figure 1 The N-terminal *myc*-tag and the K18/24R substitutions of Pex5p interfere with the polyubiquitination but do not hamper monoubiquitination. **(A)** *myc*Pex5p and Pex5pK18/24R complement the protein import defect of *pex5*Δ-cells. The strains indicated were examined for the intracellular localization of the GFP-PTS1 by fluorescence microscopy. Structural integrity of the cells is documented by bright-field microscopy. Scale bar, 5 µm. **(B)** Binding assays were performed with Pex5p-, *myc*Pex5p- or Pex5pK18/24R-containing cytosol and membranes from *pex5*Δ cells. For the export reaction, Pex5p-containing membranes were incubated with *pex5*Δ cytosol in presence of an ATP-regenerating system. Samples were analyzed by immunoblot analysis with antibodies against Pex5p. P, membrane pellet; S, supernatant. **(C)** Polyubiquitinated forms of Pex5p were visualized in samples derived from trichloroacetic acid (TCA) lysates of *pex1Δpex5Δ* and *pex4Δpex5Δ* mutant cells by immunoblot analysis. The pattern of higher molecular-weight forms indicated the poly-ubiquitination of wild-type Pex5p that is typical for mutants that are affected in late stages of the import pathway. In contrast, neither *myc*Pex5p nor Pex5pK18/24R was polyubiquitinated in these mutants. **(D)** Monoubiquitinated Pex5p and *myc*Pex5p or Pex5pK18/24R was visualized by preparation of indicated cell-lysates in the presence of N-ethylmaleimide (NEM) to prevent deubiquitination. Membrane enriched fractions were subjected to TCA-precipitation and immunoblot analysis. Co-expression of *myc*-Ub resulted in a shift of modified Pex5p demonstrating that these bands represent ubiquitinated Pex5p-forms as demonstrated earlier for the wild-type proteins [4, 5]. In contrast, no band shift to a lower molecular weight was observed when polyubiquitination was prevented by expression of UbK48R thereby confirming the mono-ubiquitination.

Figure 2 Monoubiquitination of Pex5p depends on the presence, activity and peroxisomal localization of the ubiquitin-conjugating enzyme Pex4p. **(A)** *myc*Pex5p was transformed into UBC mutant strains and isolated by immunoprecipitation. In contrast to all other tested *ubc*-deletion-strains, no mono-Ub-*myc*Pex5p is observed in the *pex4Δ* strain. **(B)** Equally**,** monoubiquitination of Pex5pK18/24R was not observed in a strain expressing a catalytical inactive Pex4p or in *pex22*Δ cells that lack the membrane anchor of Pex4p. **(C)** Pex4p-mediated monoubiquitination of Pex5p occurs upstream of the AAA-ATPase complex. *myc*Pex5p isolated from indicated strains by immunoprecipitation was tested for Pex5p-modification by immunoblot analysis. Monoubiquitination of *myc*-Pex5p still took place in the *pex1*Δ strain, but not in *pex1*Δ/4Δ double deletion strain, indicating that the presence of the AAA-peroxin is not required for receptor-modification.

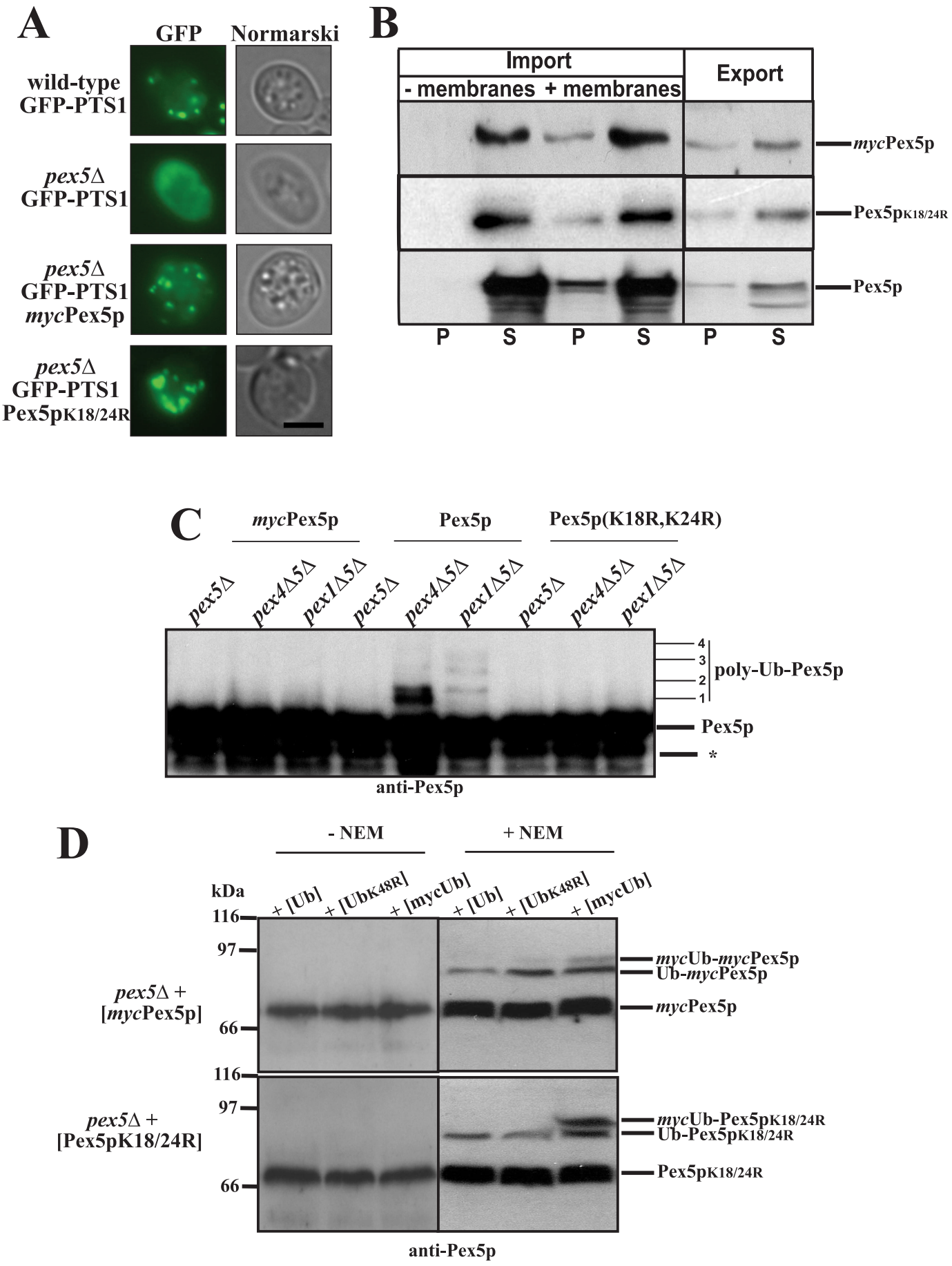
The presence of Pex5pK18/24R and *myc*Pex5p was assayed by immunoblot analysis using *myc*- or Pex5p-antibodies.

Figure 3 Receptor release requires ubiquitination. **(A)** *In vitro* export assays were performed with membranes from *pex1Δ*, *pex4Δ* or *pex1Δ4Δ* cells expressing wild-type Pex5p, Pex5p(K18/24R) or *myc*Pex5p and cytosol-fractions as indicated. Cytosolic supernatant (S) and pellet (P) fraction were analyzed for the presence of Pex5p. Under *PEX4*-deficient conditions, the ATP- and AAA-peroxin dependent export of wild-type Pex5p was significantly reduced. No export was observed when *PEX4*-deficiency was combined with Pex5p-foms lacking polyubiquitination.

Figure 4 Model for the ubiquitination and recycling of the PTS1-receptor Pex5p. Proteins harbouring a peroxisomal targeting signal of type 1 are recognized by the import receptor Pex5p in the cytosol. Upon association of the cargo loaded receptor with the docking complex

subunits, at least a fraction of Pex5p inserts into the bilayer and cargo is released into the peroxisomal lumen (not shown). The membrane bound Pex5p can be modified by poly- as well as monoubiquitination with the polyubiquitination being dependent on the E2 enzymes Ubc4 and Ubc5. Polyubiquitinated Pex5p is recognised by the AAA-peroxins Pex1p and Pex6p and directed to proteasomal degradation. This is supposed to be part of a quality control system that withdraws Pex5p from the import cycle. Monoubiquitination of Pex5p is mediated by the E2 enzyme Pex4p which is then recognized by the AAA peroxins Pex1p and Pex6p and released from the peroxisomal membrane to the cytosol, where it is made available for further rounds of matrix protein import.

Supplementary Figure 1. **(A)** Monoubiquitination of Pex5p depends on the activity and peroxisomal localization of the ubiquitin-conjugating enzyme Pex4p. *myc*Pex5p was transformed into UBC mutant strains and isolated by immunoprecipitation. No mono-Ub-*myc*Pex5p is observed in the *pex4Δ* strain expressing a catalytical inactive Pex4p or in *pex22*Δ cells that lack the membrane anchor of Pex4p. The presence of *myc*Pex5p was assayed by immunoblot analysis using *myc*- or Pex5p-antibodies. **(B)** Pex4p-mediated monoubiquitination of Pex5p occurs upstream of the AAA-ATPase complex. Whole cell lysates prepared from cells expressing Pex5pK18/24R were tested for Pex5p-modification by immunoblot analysis with Pex5p-antibodies. Monoubiquitination of Pex5pK18/24R still took place in the πεξ1Δ strain, but not in *pex1*Δ/4Δ double deletion strain, indicating that the presence of the AAA-peroxin is not required for receptor-modification.



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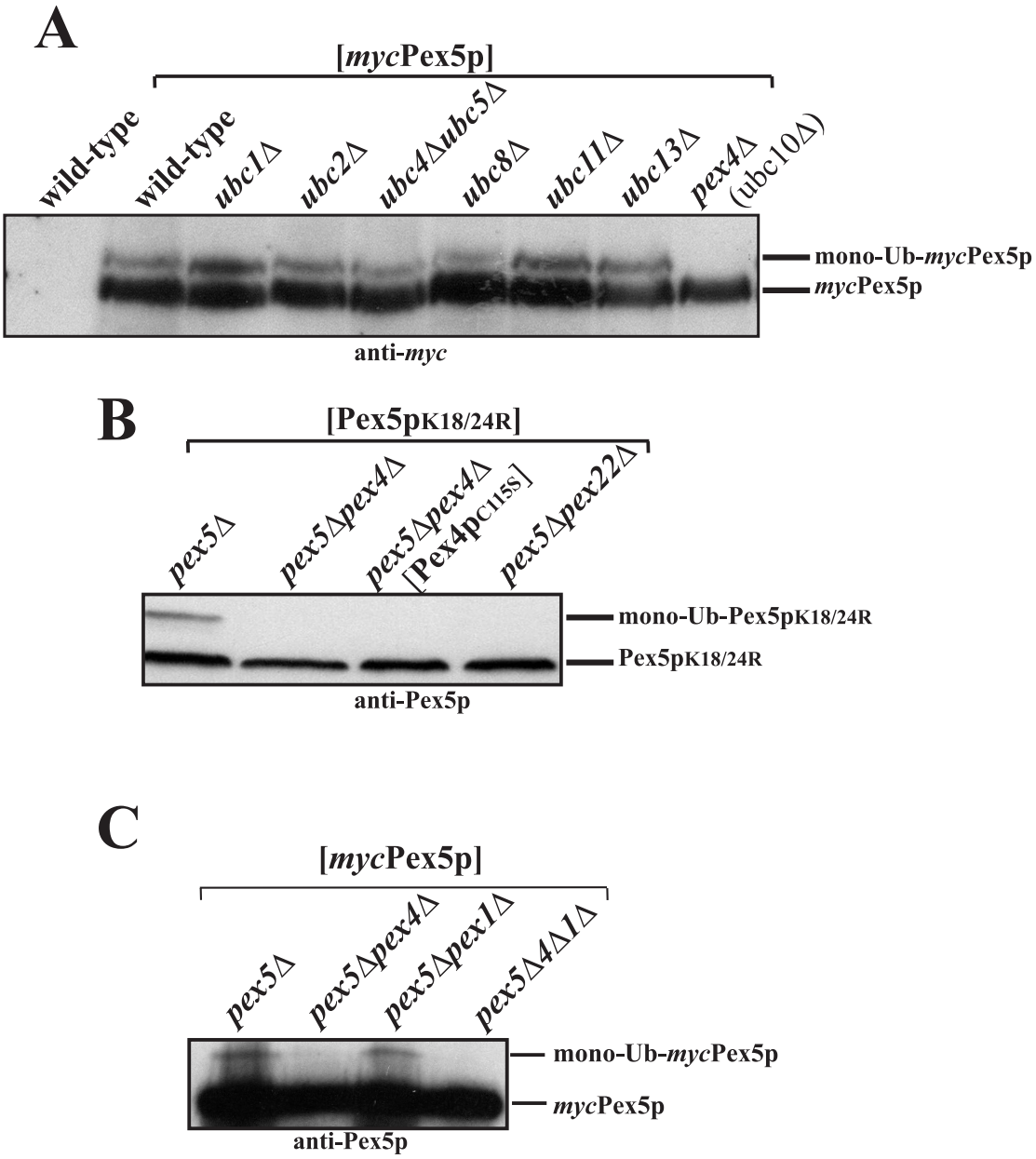

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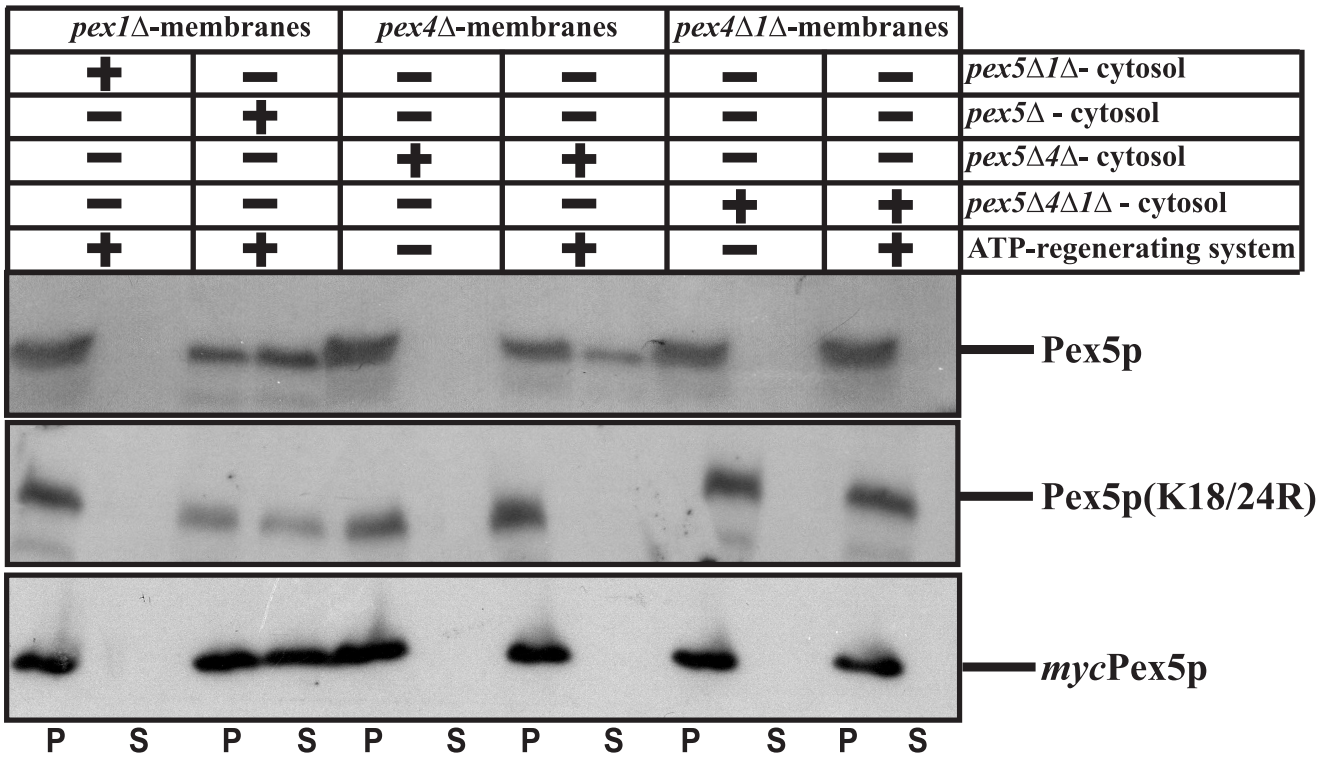

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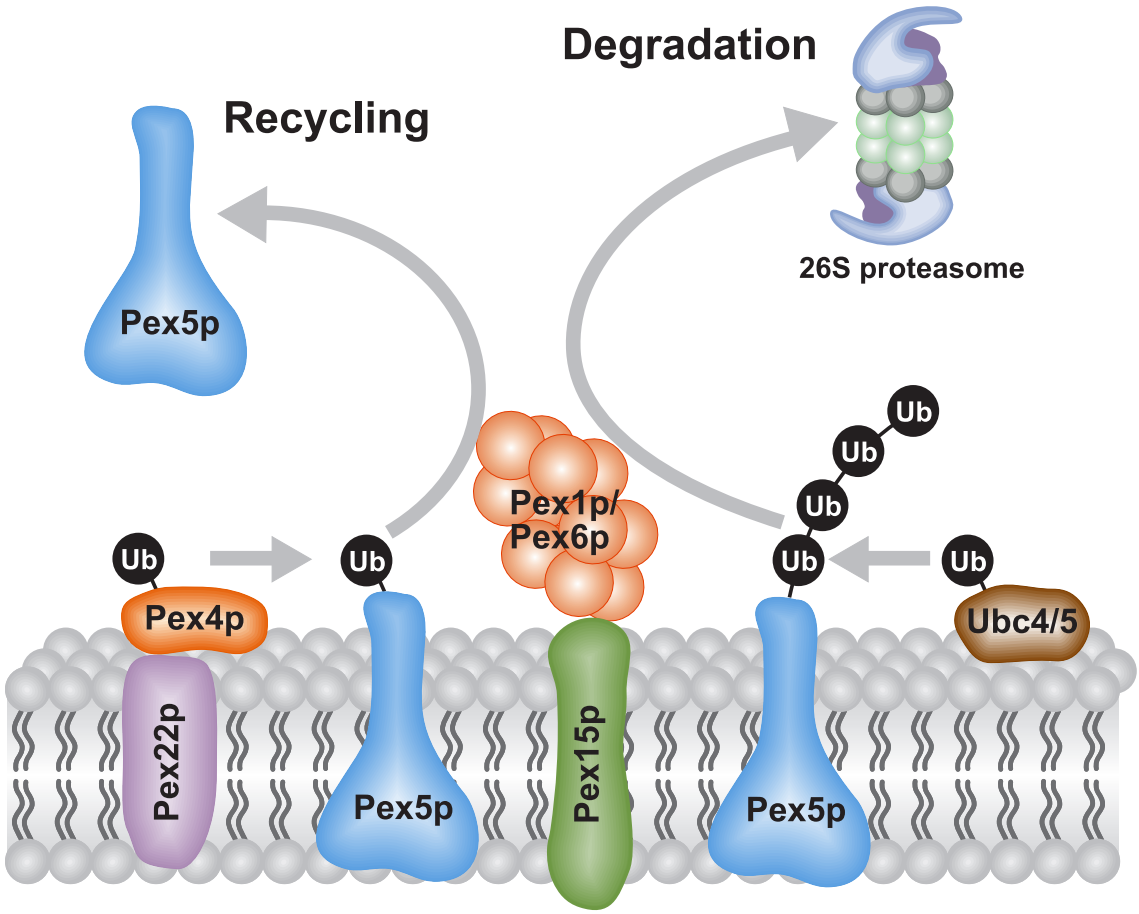

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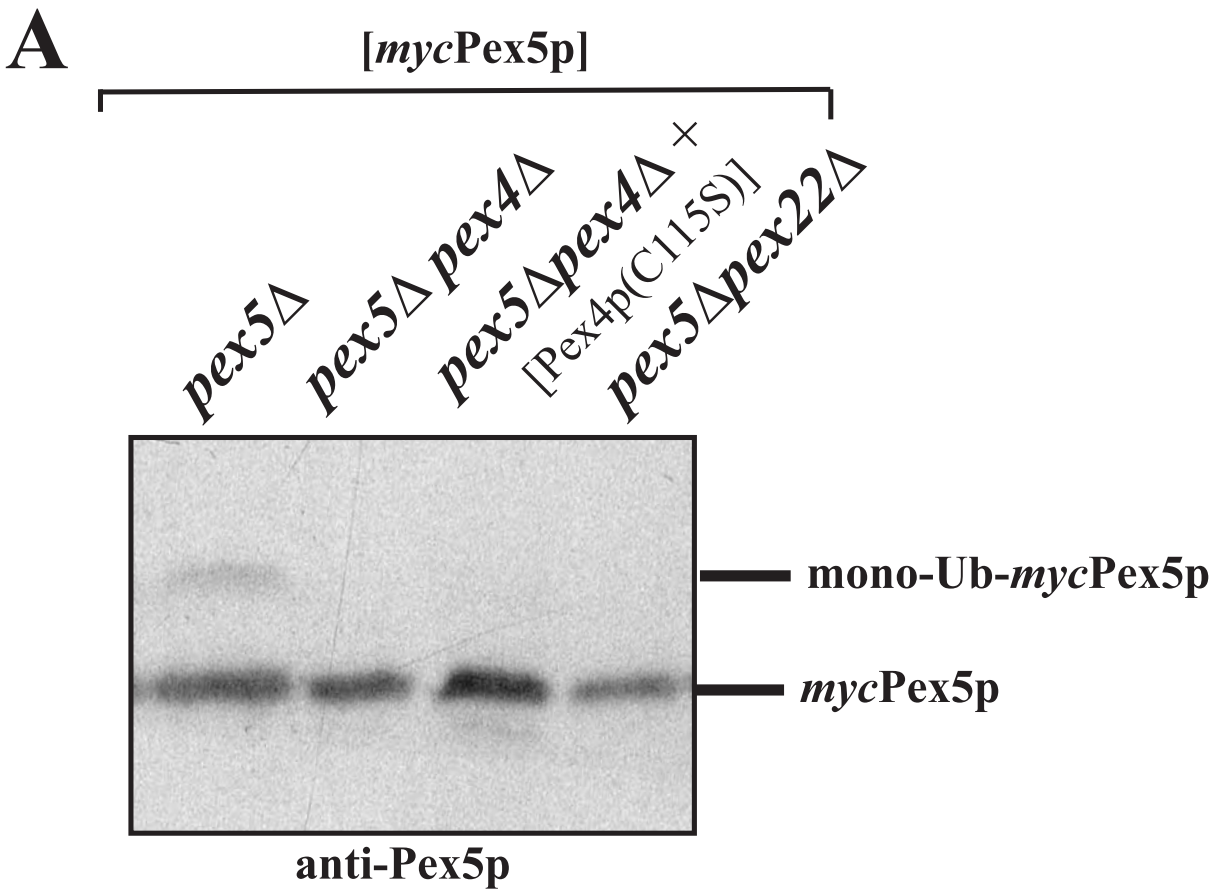

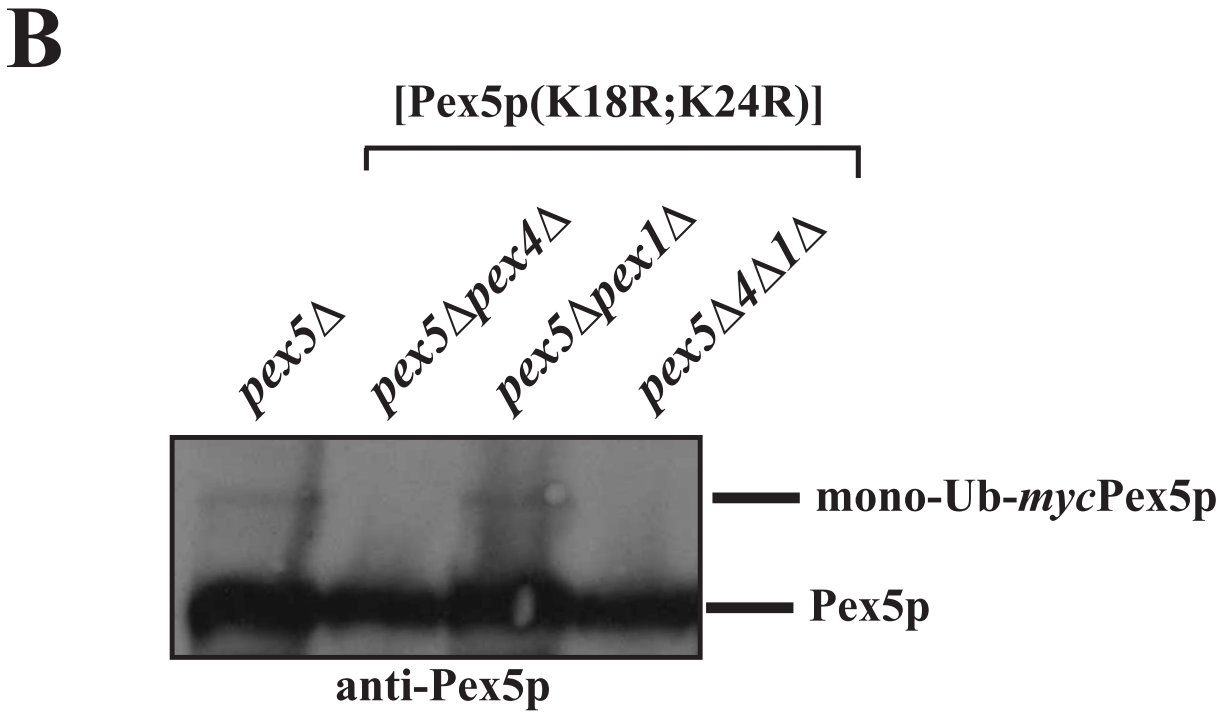

Erdmann_Supplementary_Figure 1

2.5

Function of the ubiquitin-conjugating enzyme Pex4p and the AAA peroxins Pex1p and Pex6p in peroxisomal protein transport.

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Function of the ubiquitin-conjugating enzyme Pex4p and the AAA peroxin complex Pex1p/Pex6p in peroxisomal matrix protein transport

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Introduction

Peroxisomes are cellular organelles present in all eukaryotic cells. They compartmentalize many enzymes involved in lipid metabolism and defence against oxidative stress (1) (2). The importance of peroxisome-specific metabolic processes is reflected by human disorders associated with peroxisomal defects (3). These genetically determined disorders can be categorised as (a) disorders resulting from a defect in a single peroxisomal metabolic enzyme like the X-linked adrenoleukodystrophy or (b) disorders that result from a deficiency in the biogenesis of the peroxisome, referred to as peroxisome biogenesis disorders (PBDs) with the Zellweger syndrome being the best known example. The incidence of PBDs is rather low, however, there is no cure to date these fatal diseases which normally lead to the death of the affected children within their first year of life.

Peroxisomes were first described by Rhodin (1954) (4) as `microbodies` in mouse kidney. Subsequently, de Duve (1966) (5) isolated an organelle fraction containing catalase and H_2O_2 producing oxidases which he named peroxisomes. During the following two decades, advances in our understanding of the biochemistry of peroxisomes and their important role in β-oxidation (all organisms), glyoxylate cycle (plants and fungi), photorespiration (plants), ether lipid and cholesterol biosynthesis (mammals) and glycolysis (trypanosomatids) were achieved (6) (7) (3) (8). This pronounced variability in enzyme content and thus metabolic function marked peroxisomes as multi-purpose organelles. Additionally, they vary in morphology, size and number depending on the species or cell type. The study of peroxisomal biogenesis and protein import was hampered for a long time by the low abundance of peroxisomes in many tissues and their great fragility. This changed when it became clear that peroxisome proliferation could be induced by manipulation of the carbon source in baker`s yeast (9). When offered a fatty acid as the sole carbon source, peroxisomes are required for growth because they are the exclusive site for fatty acid degradation in yeast.

This allowed the screening and identification of mutants in peroxisome function with genetic approaches (10,11). Mutants affected in peroxisome biogenesis are phenotypically characterized by the mislocalisation of peroxisomal matrix proteins and referred to as *pex* mutants, which identifies the corresponding gene as a *PEX* gene and the protein it encodes as a peroxin (12). To date 32 peroxins are known (13). They are involved in the three key stages of peroxisomal development: (1) formation of the peroxisomal membrane (import of peroxisomal membrane proteins), (2) compartmentalization of peroxisomal matrix enzymes (import of peroxisomal matrix proteins) and (3) peroxisome proliferation.

This chapter will focus on the enzymatically catalyzed mechanisms underlying transport of matrix proteins across the peroxisomal membrane into the lumen of the organelle, a process, which involves most of the known peroxins.

I. Peroxisomal matrix protein import

Peroxisomes are surrounded by a single membrane and lack DNA. This means, that their proteins are encoded in the nucleus, and the matrix proteins and most of the membrane proteins are synthesized on free ribosomes and imported posttranslationally (reviewed in (14)), while accumulating data indicate that the peroxisomal membranes and a subset of the peroxisomal membrane proteins might derive from the endoplasmatic reticulum (reviewed in (13)). After formation of the protein import machinery at the peroxisomal membrane, the matrix proteins are recognized by dynamic receptors in the cytosol and directed to the peroxisome. According to the model of shuttling receptors, the receptor/cargo complex reaches the luminal side of the peroxisome, where the complex is disassembled in order to release the cargo and return the receptor to the cytosol.

(1) Import of folded and oligomeric proteins across the peroxisomal membrane

Another characteristic feature of peroxisomes is the fact that they can import fully folded and even oligomeric protein complexes. This distinguishes them from the well established translocons of mitochondria, chloroplasts and the endoplasmatic reticulum, which import unfolded polypeptides only. One example is catalase that can cross the peroxisomal membrane as a tetramer (15). The abundant matrix protein alcohol oxidase had been shown to be imported in *Candida bondinii* as fully folded monomers that form homo-octameric complexes soon after import (16). Another example is acyl-CoA oxidase, which was shown to be imported as a pentameric complex in *Yarrowia lipolytica* (17).

(2) Sequential model for PTS-receptor cycle

The import of matrix enzymes is accomplished by receptors that shuttle between the cytosol and the peroxisomal compartment. In the following, we will briefly summarize the basic steps of this receptor cycle (Fig. 1).

Receptor-substrate binding

In principle, proteins destined for import into peroxisomes are targeted via two pathways that rely on two conserved peroxisomal targeting signals. The majority of peroxisomal matrix proteins possess a PTS1 at the very carboxy-terminus consisting of the tripeptide SKL sequence or species-specific variants (18). Pex5p interacts with the signal via six tetratricopeptide (TPRs) within its carboxy-terminal half (19). The PTS2 is found near the amino-terminus of only a few matrix proteins and has the consensus sequence (R/K)/(L/V/I)X5(H(Q)(L/A) (20). PTS2-containing proteins are recognized by the WD40 protein Pex7p (21,22). Pex7p cooperates with the Co-receptors Pex18p and Pex21p in *S. cerevisiae* (23) or its orthologues Pex20p-like proteins in different yeast and fungi species

(24-27) in order to form the receptor-cargo-complex. In mammalian cells, a short and a long isoform of Pex5p have been identified, termed Pex5S and Pex5L (28,29). These proteins differ only in a short insertion, which has been demonstrated to bind Pex7p. Furthermore, it has been shown that PTS1 and PTS2 pathways are also coupled in plants (30) (31). Consequently, while in yeast und fungi both PTS pathways share the same membrane bound protein import machinery, they converge in higher eukaryotes already at the level of Pex5L (32).

Docking and membrane association of the receptor/cargo-complex

The docking complex of the peroxisomal import machinery for matrix proteins comprises the three peroxins Pex13p, Pex14p and Pex17p. Pex17p is a peripheral membrane protein which associates to peroxisomes via Pex14p (33). Pex13p is an integral membrane protein which contains a Src homology (SH3) domain that directly binds Pex5p as well as Pex14p whereas the N-terminal region is required for interaction with Pex7p (34). Pex5p and Pex20p contain Wxxx(F/Y) repeats that have been demonstrated to be needed for interaction with the docking factors (27,35-37). The amount of Pex5p, Pex7p and Pex20p that can be found at peroxisomal membrane remnants is dramatically reduced in *pex14*Δ mutant cells in comparison to *pex13*Δ, *pex12*Δ or wild-type membranes (29). Additionally cargo-loaded Pex5p exhibits a higher binding affinity to Pex14p than to Pex13p (38). Thus the interaction to Pex14p is believed to be the entrance for Pex5p to the complex network of protein-protein interactions at the peroxisomal membrane.

Translocation and cargo release

The exact mechanism underlying translocation and the components of the translocon have not yet been identified. The possibility is discussed that components of the docking subcomplex themselves are part of the translocon (39). The multiple binding sites for Pex5p at the

peroxisomal membrane might reflect the existence of an import cascade where cargo-loaded receptor interacts with different components of the import machinery (40).

One interesting fact about Pex5p is that it changes its membrane topology during protein import cascade, as it behaves like an integral membrane protein in concert with Pex14p (41). Finally, Pex5p enters the lumen of the peroxisome (42), although it is still a matter of debate if the whole receptor-cargo-complex ("extended shuttle hypothesis") or just a part of Pex5p ("simple shuttle hypothesis") does see the matrix during cargo release (43). Pex7p has been demonstrated to behave like a cycling receptor as well (44). Recently, a model was postulated drawing parallels to pore forming toxins and suggesting that a population of Pex5p itself forms the dynamic import pore via protein-lipid interactions thereby opening the membrane dynamically for a cargo-loaded second Pex5p species (39).

The exact mechanism of cargo release inside the peroxisome is not understood. The functional role of the intraperoxisomal Pex8p, which contains both the PTS1- as well as the PTS2-sequence that may function in disassembling the receptor/cargo complex, is discussed controversly (45,46).

Receptor release to the cytosol

Following to cargo liberation, Pex5p, Pex7 and Pex20p are exported to the cytosol again. This process may need the peroxins Pex2p, Pex10p and Pex12p, which are RING-(*really interesting new gene*)-finger containing proteins. This model is based on the observation that Pex5p and Pex20p accumulate in the peroxisomal lumen in RING finger mutants (27,47-49). This RING finger complex itself is linked to the docking complex via Pex8p in *S. cerevisiae* (47).

Pex4p is an ubiquitin-conjugating enzyme (50) which is anchored to the peroxisomal membrane via Pex22p (50-52). An epistasis analysis placed Pex4p and Pex22p at the end of the Pex5p receptor cycle together with the AAA peroxins Pex1p and Pex6p (53). The AAA-

(*ATPases accociated with various cellular activities*)-peroxins form a complex in the cytosol (54) and are linked to the peroxisomal membrane via Pex15p in *S. cerevisie* (55) or the orthologues Pex26 in mammalian cells (56). Deletion of these components leads to an accumulation of Pex5p at the peroxisomal membrane and impairs Pex5p export *in vitro* in cells of *S. cerevisiae* (57) and human patients (58). After remerging to the cytosol, the receptors are available for further rounds of protein import.

Whereas the import of Pex5p appears to be based on binding-affinity mediated protein-protein or, according to the "transient pore model", additional protein-lipid interactions (39), the export relies on three enzymatic activities: ubiquitin-conjugating enzymes (Pex4p), ubiquitin-protein isopeptide ligases (putatively the RING-finger peroxins), and ATPases (the AAA peroxins Pex1p and Pex6p).

II. Overview: Enzymes involved in ubiquitination

(1) Enzymatic cascade for protein modification

Ubiquitination takes place as an enzymological cascade requiring the sequential activity of three types of enzymes. The ubiquitin-activating enzyme (Uba or E1) hydrolyzes ATP and forms a high-energy thioester bond between the cysteine of its active site and the carboxy-terminal Gly76 of ubiquitin, thereby activating the COOH-terminus of ubiquitin for nucleophilic attack. Activated ubiquitin is then passed on to an Ub-conjugating enzyme (Ubc or E2) by transthiolation. Finally, ubiquitin is covalently attached to the ε-amino group of a target lysine residue within the substrate protein through a isopeptide bond. This last reaction is mediated by an ubiquitin-protein isopeptide ligase (E3), which provides substrate specificity for this reaction, as it binds directly to the target protein. While HECT (*homologous to E6-AP C-terminus*)-E3 enzymes can form a thioester with ubiquitin

themselves before substrate attachment, RING- and U-box E3s do not seem to interact with ubiquitin and primarily serve as platforms to coordinate E2 and substrate (59). The organization of this reaction sequence is hierarchical: Most species contain only one single E1 enzyme, while in yeast 11 and in humans about 100 E2 enzymes are known. The number of ubiquitin-ligases is still growing and is anticipated to be around 50 in yeast and about 1000 in human cells (60).

The types of ubiquitin modifications that can form are diverse. In the simplest form, one single ubiquitin molecule is attached, which is defined as monoubiquitination. Alternatively, several lysine residues can be charged with single ubiquitin molecules, giving rise to multiple monoubiquitination (`multiubiquitination`). Since ubiquitin contains seven lysine residues itself, ubiquitin molecules can become substrates for ubiquitin-ligation themselves and are able to form different types of chains in a process known as polyubiquitination.

(2) Downstream components of ubiqutin-based protein targeting systems

Ubiquitin is an inducible and reversible signal. The different kinds of ubiquitin modifications function as signalling dependent devices for regulating cellular functions (61). Modification of target proteins with polyubiquitin chains linked via K48 are the best characterized to date. This modification serves as the classical degradation signal for cytosolic and organellar proteins as K48-branched chains of at least four ubiquitin-molecules are recognized by the 26S proteasome. K63-linked chains and monoubiquitination are involved in internalization during endocytosis of plasma membrane receptors. Monoubiquitin is further associated with membrane protein uptake in late endosome vesicles for delivery to the lysosome or vacuole (62). Especially (multiple) monoubiquitination events are linked to a wide range of non-proteolytical processes by serving as targeting signals (63). Examples include p53 or Rad18 that alternatively need to be monoubiquitinated for export from the nucleus or they are polyubiquitinated for proteasomal degradation (61,64,65). Monoubiquitination of histones is

required for mitotic cell growth and meiosis and regulation of eukaryotic transcription activation (66). DNA repair (nucleotide excision repair) is linked to transient posttranslational modifications of PCNA, which is marked by monoubiquitination, a K63-linked polyubiquitin chain or by the ubiquitin-like protein SUMO at the same lysine residue (67).

The different forms of modification are recognized by proteins containing specialized ubiquitin-binding domains (UBDs) that non-covalently bind to ubiquitin and thus function as downstream components of the ubiquitin-targeting system. They all have different ubiquitin-binding affinities, depending on the nature of the ubiquitin modification (60). The conjugation of ubiquitin to its target protein is a reversible process. Removal of ubiquitin is mediated by deubiquitinating enzymes (DUBs). Besides recycling of free ubiquitin molecules, DUBs function in stabilization of proteins or quenching of ubiquitin-based targeting signals (68).

III. The ubiquitin-conjugating enzyme Pex4p in peroxisome biogenesis

(1) Ub-conjugating enzymes of the Ubc4p family involved in PTS receptor regulation

Ubiquitin-conjugating enzymes play a central role in the process of ubiquitination. They function to bridge the first, non-specific step of ubiquitin activation by E1 with the transfer of activated ubiquitin to target-proteins by substrate-specific E3-enzymes.

Peroxisomal biogenesis has been implicated with ubiquitination ever since the discovery of the ubiquitin-conjugating enzyme Pex4p in 1992 (50), which has also been classified as Ubc10p (59). Recent studies identified the Ubc1p/Ubc4p/Ubc5p-familiy to be involved in peroxisomal processes in *S. cerevisiae* as well (69-72). Members of this group are particularly well conserved in higher metazoans, e.g. being represented by the UbcH5-family in humans. They show a capacity to interact with a wide range of E3 enzymes from both HECT and

RING finger families and, depending on the specific activity of the E3 enzyme in question, are capable to facilitate mono- or polyubiquitination via K29, K48 or K63 of different proteins. This ability allows them to be involved in both proteasomal and lysosomal degradation processes (73).

PTS1 receptor polyubiquitination

The cycling PTS1 receptor Pex5p has been shown to be a substrate for Ubc4p (69-72). Catalyzing the formation of K48-linked polyubiquitin chains, Ubc4p marks the PTS1-receptor for proteasomal degradation. In *UBC4*-deletion strains, this function can be taken over by Ubc5p (69) and Ubc1p (71), which can be explained by the fact that these enzymes chare a sequence similarity of over 90% (74-76).

Polyubiquitination of Pex5p has been demonstrated to take place at the peroxisomal membrane at the end of the receptor cycle, as Pex5p has to pass the docking- and the RING-finger complexes before it can be modified. These modified receptor molecules are targeted to the 26S proteasome as polyUb-Pex5p accumulates in strains with temperature sensitive mutants of the 19S-cap (*cim3-1*, *cim5-1*) *in vivo* (69) (72) or after treatment of lysates from wild-type cells with MG132 *in vitro* (57). Current evidence suggests that Pex5p polyubiquitination in *S. cerevisiae* is not essential for Pex5p function in peroxisomal protein import under physiological conditions. More likely, it seems to be a part of a quality control system that withdraws a fraction of the membrane-accumulated Pex5p that has got stuck in the export pathway by targeting it to the proteasome. Alternatively to “quality control”, the term RADAR (*receptor accumulation and degradation in absence of recycling*) has been suggested (27). This mechanism is supposed to reopen binding capacities for cargo-loaded receptors at the peroxisomal membrane. However, it can be imagined that failure in the removal of Pex5p designated for disposal would affect matrix protein import. In fact, while

deficiency in Ubc4p or Ubc5p alone can be compensated, the deletion of both leads to a partial import defect of peroxisomal matrix proteins (69).

Polyubiquitinated species of Pex5p accumulate in *S. cerevisiae* cells lacking `late` import pathway peroxins Pex1p, Pex4p, Pex6p, Pex15p and Pex22p, making them detectable even without the inhibition of the proteasome. Apparently the efficiency of proteasomal disposal of the receptor varies among different species drastically. While *Sc*Pex5 is heavily polyubiquitinated in mutants that are supposed to block receptor recycling, single deletions in the same set of proteins result in a dramatic decrease of the Pex5p steady state concentration in human cells (49,77), plants (78), *Hansenula polymorpha* (79-81) or *Pichia pastoris* (53). The phenomenon of PolyUb-Pex5p accumulation in *S. cerevisiae* facilitated the discovery and functional characterization of Pex5p ubiquitination and provides the long-sought for explanation for the instability of Pex5p in human cell lines (77).

PTS2 Coreceptor polyubiquitination

Ubiquitination plays an important role in regulation of the PTS2-receptor pathway. In contrast to Pex5p, which facilitates PTS1-protein import on its own, the PTS2-receptor Pex7p is associated with auxiliary proteins. *Saccharomyces cerevisiae* possesses the two redundant Co-receptors Pex18p and Pex21p (23).

While the PTS1-receptor Pex5p is a stable protein with a life-span of over five hours under wild-type conditions (71), the PTS2-coreceptors Pex18p and Pex21p exhibit a half-time of only about ten minutes (70). This rapid turnover may be closely connected to the physiological function of these proteins. Their degradation takes place in the 26S proteasome and is mediated by Ubc4p and Ubc5p (70). In *ubc4*Δ*ubc5*Δ mutant cells, Pex18p is still polyubiquitinated by Ubc1p but the efficiency of this process is reduced (Platta, unpublished

results). Interestingly, Pex18p accumulates in mutants of the membrane standing components of the peroxisomal protein import machinery, e.g. *pex13*Δ, *pex2*Δ or *pex1*Δ. This stabilisation and accumulation is reverted if additionally *PEX7* is disrupted, which identifies Pex7p as a stabilizing factor for Pex18p at the peroxisomal membrane. Based on coimmunoprecipitation studies, apparently not all Pex7p forms a complex with Pex18p while the entire pool of Pex18p seems to be saturated with Pex7p (70). These results indicate that the Ubc4p-mediated turnover of Pex18p is associated with its normal function during matrix protein import rather than being an abortive degradation.

The family of PTS2-coreceptors is evolutionary divergent. While *S. cerevisiae* contains Pex18p/Pex21p and human as well as plant cells harbour the Pex5L, most other yeasts and fungi have Pex20p-like proteins. The Pex18p/Pex21p orthologue Pex20p displays interesting parallels to the PTS1-receptor Pex5p in terms of its Ubc-based regulation. In contrast to Pex18p and Pex21p, which exhibit a turnover rate of ten minutes, Pex20p of *Hansenula polymorpha* is stable for at least 60 minutes (26) and is not destabilized in the absence of Pex7p in *Pichia pastoris* (27). A further similarity to Pex5p is that Pex20p of *P. pastoris* is polyubiquitinated with K48-branched chains in the absence of either Pex4p, Pex1p or Pex6p (27).

Conserved regulatory mechanism

Although the degree of functional regulation of the PTS-receptors in general may differ on the cell biological level, the basic biochemical recognition signals and mechanisms are the same.

It has been known that the carboxy-terminal half of Pex5p, which contains the TPR-motifs, serves as binding region for the PTS1-cargo proteins, while the amino-terminal half contains the peroxisomal targeting function, docking-complex-interaction sites made of diaromatic pentapeptide repeats (Wxxx[F/Y]), and the yet not fully defined putative export signal within

the first 20 amino acids (82). The PTS2-receptor-complex seems to be functionally divided in a comparable manner in that Pex7p binds the PTS2-cargo and the co-receptors seem to contain the structural requirements for the membrane bound steps of receptor cycle (82-85). One striking example is a chimeric protein consisting of Pex18p (without it`s Pex7p binding site) fused to the TPR domains of Pex5p, which is still able to mediate PTS1-protein import (85). Amino acid sequence alignments of the N-termini of Pex5p, Pex18p, Pex21p and the Pex20p-proteins reveal that the very first lysine residue of each receptor is evolutionary highly conserved between these molecules. Experimental evidence has been provided from studies of *H. polymorpha* Pex5p (86) and *P. pastoris* Pex20p (27) that this lysine residue indeed is the acceptor of the polyubiquitin chain. Taking these findings together, an evolutionary conserved biochemical basis for Ubc4p-mediated PTS-receptor ubiquitination can be defined.

(2) Function of Pex4p/Ubc10p in receptor recycling

Pex4p/Ubc10p is an ubiquitin-conjugating enzyme essential for peroxisomal biogenesis, which defines it as the only ubiquitin-conjugating enzyme known to be indispensable for the biogenesis of an organelle. The molecular function of this enzyme has been a mystery since its initial description in 1992 (50).

Pex4p in peroxisomal biogenesis

Pex4p is anchored to the peroxisomal membrane via its interaction to the membrane integrated Pex22p in yeasts and plants (51,52). This interaction is needed for Pex4p function as *pex22*Δ strains are characterized by the complete mislocalization of Pex4p to the cytosol and they share a similar phenotype like *pex4*Δ cells.

Pex4p contains the catalytically relevant active site cysteine residue of ubiquitin-conjugating enzymes within the core UBC fold (50,87). Site directed mutagenesis of the Cys residue results in a loss of Pex4p activity as the point mutant exhibited the same phenotype as the *pex4*Δ deletion strain, while it was still attached to peroxisomes (50). This Cys residue is needed to form a thioester bond to ubiquitin, as this Ub-Pex4p conjugate could be detected *in vivo* under non-reducing conditions only and was disrupted under reducing conditions or in a Cys to Ala point mutated Pex4p (87). Loss of Pex4p ubiquitin-conjugating activity is closely connected to an import defect of peroxisomal matrix proteins. This argues for a direct role of Pex4p mediated ubiquitination in receptor cycle during matrix protein import. Indeed, Pex5p stability has been found to depend on the presence and activity of Pex4p, as the Pex5p level drops significantly in PEX4 or PEX22 mutants in humans and different yeasts, with the exception of *S. cerevisiae* where polyubiquitinated Pex5p accumulates at the peroxisomal membrane (69,72). Instability in *pex*-mutants was also observed in *P. pastoris* in the case of Pex20p (27) which was used as a tool to examine the sequence of Pex5p interactions at the peroxisomal membrane (53). This epitasis study placed Pex1p and Pex6p downstream of the RING-finger complex and found Pex4p and Pex22p to act even later at the very end of the receptor cycle, as double deletions of Pex1p and Pex4p displayed a *pex1*Δ -like phenotype. Similar experiments in *S. cerevisiae* showed a mixed phenotype (namely a combined PolyUb-pattern of *pex1*Δ and *pex4*Δ) and indicated the Pex5p interaction sequence to be branched spatially after the RING complex ((69)and unpublished data). Studies in *pex4*Δ strains of *H. polymorpha* found residual amounts of Pex5p to accumulate inside of peroxisomes, indicative for a functional connection of Pex4p and receptor release. The mislocalisation of PTS1-matrix proteins to the cytosol in a *PEX4* deletion strain can partially be restored by a massive overexpression of Pex5p (80). This observation strongly suggests that it is not the physical interaction between Pex4p and Pex5p but the Pex5p-specific activity of this Ubc which is supposed to be a regulatory device involved in protein import into peroxisomes.

Model I.: Monoubiquitination

The finding that the deletion of an ubiquitin conjugating enzyme results in a specific polyubiquitination and degradation of its potential substrates argues for a ubiquitin-dependent, physiological and non-proteolytic role of this modification. Interestingly, Pex5p has been reported to be monoubiqutinated at two different lysine residues in wild type cells of *S. cerevisiae* (71). Ubc4p is not required for Pex5p monoubiquitination, which has also been demonstrated to be the case for Ubc1p, Ubc5p and Ubc8p, raising the attractive possibility that Pex4p may be the responsible E2 enzyme. Like the Ubc4p-dependent polyubiquitination, the monoubiquitination event takes place after docking and is located at or after the RING-finger complex. Pex5p is only transiently modified, as the monoubiquitinated Pex5p forms can only be detected by treatment with thiol-alkylating reagent NEM (*N-ethylmaleimide*), supposed to inhibit deubiquitinating enzymes, which are cysteine-proteases in yeasts. As monoUb-Pex5p is exclusively found at the peroxisomal membrane, it is proposed to be deubiquitinated under wild-type conditions prior or during export back to the cytosol. These results gave rise to the still hypothetical idea that Pex4p monoubiquitinates the PTS1-receptor to prime it for release to the cytosol (39).

Model II.: Polyubiquitination

Two recent studies report data which argue for a polyubiquitination-activity of Pex4p. Kiel and co-workers (86) noticed that obstruction of polyubiquitination by overexpression of Ub(K48R) affects PTS1 matrix protein import in the methylotrophic yeast *H. polymorpha*. Under these conditions, Pex5p is ubiquitinated and rapidly degraded by the proteasome. To test whether Ub(K48R) interferes with the function of *Hp*Pex4p, the *pex4*Δ deletion strain was complemented by massive overexpression of Pex5p as described previously (80) and in

addition, Ub(K48R) was overexpressed in parallel. Interestingly, the Pex5p-overexpression did result in a compensation of the Ub(K48R)-effect, which was considered as evidence for a Pex4p-dependent polyubiquitination of an unknown substrate involved in receptor recycling (86). The important role of K48-branched polyubiquitin chains for peroxisomal biogenesis has also been noticed in *P. pastoris* (27). Overexpression of Ub(K48R) in *P. pastoris* results in a peroxisome biogenesis defect and induces accumulation, polyubiquitination and degradation of Pex20p.

Thus, overexpression of Ub(K48R) mimics the characteristics of a defect in receptor export which might be explained by the assumption that polyubiquitination 1) might provide some kind of `rescue signal` under conditions when the physiological export signal for the receptors is not functional. As a result recycling might be hampered so that the membrane has to be cleared of cargo-unloaded receptors by the proteasome in order to open binding capacities for new cargo-loaded receptors or 2) might trigger the constitutive degradation of a yet unknown protein which functions as a repressor of receptor export or 3) might represent a physiological process concerning known parts of the export machinery itself: It has been demonstrated in other systems, that E3- as well as E2-enzymes undergo autoubiquitination reactions *in vivo* (88-91).

Another study concerns Pex4p and Pex22p from *A. thaliana* and discusses the potential role of Pex4p mediated polyubiquitination as a regulator of matrix protein composition (51). Glyoxysomes are specialized microbodies that function in early seedling development and are converted to peroxisomes in leafs when photosynthesis is initiated. Isocytrate lyase is a glyoxysome specific enzyme that usually is not anymore present in leaf peroxisomes. Interestingly, this protein remains stable in peroxisomes in the absence of Pex4p and Pex22p indicating that the proteins may be important during the remodeling of peroxisome matrix contents as glyoxysomes transition to leaf peroxisomes.

Basically, both models can be true, as Pex4p may interact with different E3-enzymes to either mono- or polyubiquitinate their substrate(s). Studies in Pex4p-affected mutants have been extremely valuable to explore the functional context of this Ubc. However, taking in account that the Pex4p/Pex22p unit is part of the peroxisomal protein import machinery, which is a multi-protein complex also consisting of docking-, RING- and AAA-subcomplexes (47,51,92,93), it might be difficult to distinguish direct from indirect effects. For future research, it therefore will be of significant importance to establish *in vitro* assays to unequivocally prove Pex5p or other proteins to be substrates for ubiquitination-reactions mediated by Pex4p. This will also require the identification of the corresponding E3 enzymes for which the peroxisomal RING finger peroxins are the best candidates.

(3) Peroxisomal RING finger proteins as putative ubiquitin-ligase-complex

The RING finger motif was first identified in the protein encoded by the *Really Interesting New Gene 1* by Freemont *et al* in 1991 (94) and has ever since been implicated in mediating protein-protein-interactions of different kinds. In recent years, it has become evident that most if not all RING-finger containing proteins have ubiquitin-protein ligase (isopeptidase) activity and act as E3-enzymes (95,96). The canonical RING finger consensus sequence has been defined as Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys/His-X_2-Cys-X_{4-48}-Cys-X_2-Cys, where X stands for any amino acid residue. Like other cysteine rich motifs, the RING finger binds Zn^{2+}-ions through its conserved cysteine and histidine residues but is set apart from the rest by its unique "cross-brace" arrangement of their two Zn-coordination sites, where Cys1/Cys2 and Cys5/Cys6 bind the first and Cys3/His4 and Cys7/Cys8 bind the second. Zn-$^{2+}$-ion. The majority of RING finger proteins fall into two subclasses, RING-HC and RING-H2, depending on the presence of a Cys or a His in the fifth Zn^{2+} coordination site (97).

The three peroxins Pex2p, Pex10p and Pex12p are peroxisomal integral membrane proteins and possess cytosollically exposed RING domains in their carboxy-termini. All belong to the

RING-HC family, but only in Pex10p both Zn^{2+}-coordination sites are well conserved. Pex2p and Pex12p contain substitutions for the conserved Cys- and His-residues in the second Zn^{2+}-coordination site. The zinc binding capacity of Pex10p was investigated and the protein has been proven to coordinate zinc with its RING domain (98). This zinc-dependent activity of the peroxisomal RING fingers could provide the basis for the general zinc requirement of matrix protein import (99). The RING finger peroxins are essential for peroxisomal biogenesis in all species analysed (100-105). They interact with each other by forming an unique trimeric RING-finger complex and are also capable of interaction with Pex5p (48,100,105-107). More than one decade after the identification of the first RING peroxin (108), the molecular function of the peroxisomal RING-finger complex is still a matter of debate. While some recent studies demonstrate that the RING peroxins are required for Pex5p import in an *in vitro* system (58) others find Pex5p (48,49,58) or the PTS2-coreceptor Pex20p (27) to accumulate inside the peroxisomal lumen in cells with disrupted RING-complex. Direct evidence for E3 ligase activity of the RING peroxins is still missing. It is important to note, however, that both mono- and polyubiquitination of Pex5p depend on the presence of the RING finger peroxins (69,71,72). Additionally, a Pex22p-dependent interaction between Pex4p and Pex10p has been observed in the split ubiquitin system (109). Thus, Pex4p and Ubc4p might be recruited to the peroxisomal membrane and exhibit their E2-activity in concert with the putative E3-ligase peroxins in order to mono- or polyubiquitinate the PTS-receptors.

IV. The AAA family ATPases

Pex1p is one of the defining members of the large AAA family of enzymes (10,110). AAA proteins are characterised by the presence of 200-250 amino-acid ATP-binding domains that contain Walker A and B motifs. AAA proteins themselves belong to the superfamily of P-loop NTPases (111).

(1) Function and structure of AAA-type ATPases

AAA proteins have an N-terminal Non-ATPase domain which is followed by either one or two AAA domains (D1 and D2). In some proteins with two AAA domains, both are evolutionarily well conserved (like in Cdc48p/97). In others, either the D2 domain (like in Pex1p and Pex6p) or the D1 domain (in Sec18p/NSF) is better conserved in evolution (Fig.2) The classical AAA has been expanded by inclusion of a number of more distantly related cellular regulators and termed AAA+ family of ATPases (112). AAA+ proteins are involved in protein degradation, membrane fusion, DNA replication, microtubule dynamics, disassembly of protein complexes and protein aggregates (111). AAAs are mechanoenzymes that manipulate the structure of substrate proteins and thereby unfold them or disassemble protein complexes.

The physiologically active form of these enzymes often is an homo-hexamer. The hexameric enzymes have an overall shape that resembles a double-ring with a central pore that might be involved in substrate processing. In the hexameric configuration, the ATP-binding site is positioned at the interface between the subunits. Upon ATP binding and hydrolysis, AAA enzymes undergo conformational changes in the AAA-domains as well as in the N-domains. These motions can be transmitted to substrate protein.

AAA proteins are not restricted to eukaryotes. Prokaryotes have AAA which combine chaperone with proteolytic activity, like the ClpAPS complex, which mediates protein degradation and recognition in *E. coli*. The basic recognition of proteins by AAAs is thought to occur through unfolded domains in the substrate protein (113). In HslU, a bacterial ClpX/ClpY homologue of the HSP100 family of AAA+ proteins, the N- and C-terminal subdomains move towards each other when nucleotides are bound and hydrolysed. The terminal domains are most distant in the nucleotide-free state and closest in the ADP-bound state. Thereby the opening of the central cavity is affected (114,115).

(2) AAA ATPases in protein transport

ER-associated protein degradation (ERAD)

The AAA-type ATPase Cdc48p/p97 is perhaps the best-studied AAA protein. Misfolded secretory proteins are exported from the endoplasmic reticulum (ER) and degraded by the ER-associated degradation pathway (ERAD) (116). Substrate retrotranslocation and extraction is assisted by the Cdc48p (Ufd1p/Npl4p) complex on the cytosolic side of the membrane where the substrate is ubiquitinated by ER-based E2 and E3 enzymes before degradation by the 26S proteasome (116).

Targeting to multivesicular bodies

Multivesicular bodies are endosomal compartments that sort ubiquitinated membrane proteins by incorporating them into vesicles. This process involves the sequential action of three multiprotein complexes, ESCRT I to III (62). Vps4p is an AAA-type ATPase involved in this MVB sorting pathway. It had originally been identified as a ”class E” vps (vacuolar protein sorting) mutant (117) and was subsequently shown to catalyse the dissociation of ESCRT complexes (118). It is anchored via Vps46p to the endosomal membrane. Vps4p assembly is assisted by the conserved Vta1p protein, which regulates its oligomerisation status and ATPase activity.

V. Pex1p and Pex6p: AAA proteins required for peroxisomal biogenesis

Two lines of research have converged into the recognition of AAA proteins as enzymes important for peroxisome biogenesis. One is the study of the energetic requirement of protein import. The other is the molecular characterisation of AAA peroxins.

(1) ATP-dependency of matrix protein import

Peroxisomal matrix protein import was shown to be ATP-dependent in several *in vitro* systems. In permeablized CHO cells, import of PTS1 proteins depends on ATP, but not on GTP or a membrane potential (119). As the peroxins Pex1p and Pex6p are the only ATPases among the known peroxins, they were considered as being responsible for the bulk ATP-requirement of peroxisomal matrix protein import. The described ATP requirement may comprise several steps in the function of the AAA peroxins.

(a) Mutations in the AAA peroxin genes represent the most frequent cause of human peroxisomal biogenesis disorders (PBDs) (120). Cells with defective Pex1p or Pex6p mislocalise peroxisomal matrix proteins to the cytosol (10,121,122). As Pex1p and Pex6p interact ATP-dependently with each other (54,81,123,124), it is important to note that the most common cause of Zellweger syndrome is a disruption of the Pex1p-Pex6p interaction caused by a point mutation in the conserved ATP-binding side of Pex1p(120,125).

(b) Based on two-hybrid data, it has been discussed, that ATP hydrolysis in the conserved domain of Pex6p contributes to the disassembly of the Pex6p-Pex15p complex (55), which indicates that the AAA peroxins interact dynamically with Pex15p.

(c) *In vitro* experiments indicated that ATP is needed predominantly for the recycling of the PTS1-receptor Pex5p rather than for its insertion into the membrane (41,126). The *in vitro* reconstitution of the complete Pex5p cycle revealed that ATP-binding and hydrolysis in the conserved domains of both Pex1p and Pex6p were needed for this reaction (57). The binding and consumption of ATP may result in conformational changes that could generate the driving force to pull the receptor out of the membrane.

(d) Additionally to their involvement in matrix protein import, Pex1p and Pex6p also play a role in peroxisome membrane fusion. In the yeast *Yarrowia lipolytica*, six peroxisomal membrane subforms have been identified that in a multistep pathway assemble into mature

peroxisomes (127). The fusion of early forms in this pathway requires ATP and the AAA peroxins, which are heterogeneously distributed over these subforms (127).

(2) Structural characterisation of the AAA peroxins

The domain structure of Pex1p and Pex6p is similar to other AAA proteins: N-D1-D2 (Fig. 2). The second AAA domain, D2, is better conserved than the first. The mouse Pex1p N-domain is the only part of AAA peroxins for which X-ray structural information is available (128). It consists of two structurally independent lobes separated by a shallow groove (Fig. 3). The structure is strikingly similar to the N-domains of other AAA proteins with two ATPase domains that have been solved: Sec18p, NSF, p97 and the archaeal Cdc48p/p97 homolog VAT (129-132). In spite of the low degree in sequencece similarity, the cleft between the subdomains is structurally conserved. The structure looks similar to the cleft in NSF, which is a binding site for alpha-SNAP. While the adaptor protein for the N-terminal domain of Pex1p is still elusive, Pex6p has been demonstrated to interact with the peroxisomal membrane protein Pex15p in yeast (55) and Pex26 in human cells (56) via the N-terminus. Pex15p/Pex26 is the membrane recruitment factor for the cytosolic AAA-complex. We have little information yet on the oligomerisation status of the AAA peroxins. They are thought to build up hexameric structures (92) but it is unclear weather they form homo- or hetero-hexamers.

(3) Similarities of the peroxisomal import machinery with ERAD components

There is striking similarity of the peroxisomal import machinery that we portrait in this review with a special focus on Pex4p and the AAA peroxins on one side and the ER associated degradation (ERAD) pathway on the other side (Fig. 4). Both pathways depend on the ubiquitination cascade starting with the general ubiquitin-activating enzyme E1 (Uba1p).

Both systems include two Ubcs that are recruited to their destination via a membrane anchor (Ubc7p-Cue1p / Pex4p-Pex22p). Involved are intergral RING finger proteins Doa10p and Hrd1p in ERAD, while peroxisomes rely on the trimeric RING finger complex Pex2p-Pex10p-Pex12p. The AAA ATPase Cdc48p/p97 as well as the AAA peroxin complex Pex1p-Pex6p provide the driving force for protein extraction from the membranes. Furthermore, the proteasome is involved in both systems. The resemblance in terms of protein equipment has recently been backed up by two studies on the evolutionary basis of this similarity (133,134). Both studies concluded that peroxisomes are of eukaryotic origin with few ancestral protein motifs in eukaryotes. While abandoning the suggestion that peroxisomes might be endosymbionts, the topological question remains open. ERAD transports proteins out of the endomembrane compartment, while in peroxisome biogenesis, matrix proteins are imported. If, however, ubiquitinated ERAD substrates are equated with the peroxisomal import receptor, both processes can be understood as a ubiquitin dependent protein dislocation from an endomembrane system (Fig. 4).

VI. Receptor ubiquitination: a link between Pex4p, AAA peroxins and protein transport?

This chapter summarizes the basic experimental evidence concerning the functional roles of Pex4p and the AAA peroxins in Pex5p recycling and matrix protein import in order to combine and discuss them in a unified model (Fig. 5).

Peroxisomal matrix protein import is an energy-dependent reaction process as hydrolysis of ATP is needed for protein translocation (119,135). This energy-consuming step was further characterized to involve the receptor cycle of Pex5p (49). Studies in a permeabilized cell system of human fibroblasts provided first evidence that Pex5p accumulated reversibly under conditions when protein transport was blocked due to the absence of ATP (49). Detailed *in*

vitro studies revealed that the binding and translocation of Pex5p itself is ATP-independent, while the ATP-dependent step concerns the export of Pex5p back to the cytosol (41). Pex14p-associated Pex5p exposes the majority of its mass into the peroxisomal lumen, suggesting that tanslocation of cargo proteins into the matrix of the organelle occurs concomitantly with the formation of the Pex5p-Pex14p membrane complex and is succeeded by the ATP-dependent export reaction (41,126,136,137). The ATP-dependent export of Pex5p might be the rate-limitting step in the protein import process. Supposedly, the binding capacities for Pex5p at the peroxisomal membrane are nearly saturated under wild type conditions so that the cargo-free receptor has to be removed from the membrane in order to keep the flow of protein import going. The most simplified idea is that one cargo-free receptor has to leave in order to enable a cargo-charged receptor to enter the peroxisomal matrix. The identity of the ATPase required for Pex5p export remained a matter of debate until recently *in vitro* systems in *S. cerevisiae* (57) and human cells (58) identified the peroxisomal AAA-ATPases Pex1p and Pex6p as the motor-proteins of Pex5p export (Fig. 5).

The ATP-binding and hydrolysis sites of the second AAA-domain of Pex1p and Pex6p proved responsible for the observed energy-dependence of the export process (57). Furthermore, membrane integrated Pex5p was disassembled from Pex14p and released to the cytosol, indicating, that the integral Pex5p population is a target for the AAA-ATPases (57).

The exact mechanism in terms of substrate recognition by the AAA peroxins is still not known. The N-terminus of membrane integrated Pex5p is exposed to the cytosol and was demonstrated to be required for *in vitro* export of human Pex5p (82). Accumulation of an N-terminally trunctated version of Pex20p at the peroxisomal membrane points to similar conditions for the PTS2-coreceptors (27). The first 20 amino acids of the PTS-receptors contain a conserved motif of unknown function (Cys-X_n-Asn-(Ala/Gly)-(Leu/Ala)), which could act as the putative binding site for Pex1p or Pex6p. However, to date no direct interaction of Pex5p with Pex1p or Pex6p was detected, despite their obvious formation of a

complex at the peroxisomal membrane (57,58,92). Thus, we also have to consider that this interaction is regulated or mediated by a third factor, leading to the more general question of how the AAA peroxins can distinguish Pex5p forms destined for dislocation from cargo-charged Pex5p species. Current evidence draws the attention to the activity of the ubiquitin-conjugating enzyme Pex4p, which is required for matrix protein import (50) and might be involved in the recycling of Pex5p (53,69,72,80) and Pex20p (27). Taking further in account that the peroxisomal RING finger complex consisting of Pex2p, Pex10p and Pex12p may function as putative E3-ligase complex needed for protein import and Pex5p release, the dislocation of the PTS1-receptor may be linked to ubquitination processes. Based on the observation that Pex5p is monoubiquitinated in wild-type cells (71), the model of a Pex4p and Pex10p mediated monoubiquitination of the PTS1-receptor with the purpose of this modification is to prime Pex5p for efficient export mediated by the AAA peroxins has been discussed (39, 71). However, the experimental evidence for this modification being required for Pex5p export is still missing. It is known, that ubiquitin-based signals can be recognized by proteins harbouring ubiquitin-binding domains (60). Based on this idea, the interaction between Pex5p and Pex1p/Pex6p could be mediated by ubiquitin. This would postulate that the AAA peroxins contain ubiquitin-binding domains or associate with adaptor proteins that can bind ubiquitin. Another possibility is based on the finding, that the N-terminal half of Pex5p is a natively unfolded domain (138). The attachment of ubiquitin to substrates can induce conformational changes within the modified protein and make formally hidden binding sites accessible (60). This mode of interaction is also discussed for the AAA-ATPase Cdc48p/p97. While it is clear that the adaptor complexes Ufd1-Npl4 or Ubx-proteins bind polyubiquitin chains, the N-terminus of the AAA-ATPase itself is able to recognize chains as well as non-modified segments of its substrates (139,140). Thus monoubiqutination of Pex5p may alter its folding state and enable a direct interaction with the AAA peroxins not via but

depending on ubquitin. As a consequence of receptor export, ubiquitin has to be cleaved off after or during dislocation by one of the many deubiquitinating enzymes (68).

The importance of an optimal PTS-receptor release is also supposed to be the reason for the above mentioned existence of a quality control system at the peroxisomal membrane (27,69,72,138), which leads to the receptor polyubiquitination and proteasomal degradation in mutants lacking peroxins involved in PTS-receptor export (69,71,72). A physiological function for polyubiquitination in peroxisomal biogenesis can be postulated from the "transient pore model" (39). In this context, it is thinkable that the pore forming Pex5p population represents a dead-end structure that is withdrawn from the recycling process and has to be removed by the ubquitin-proteasome system. Alternatively, polyubiquitination may substitute for the putative monoubiquitin-based export signal for receptor release under yet to define conditions. A similar effect has been demonstrated in the case of p53 export from the nucleus (65). Although mono- and polyubiquitination target p53 to different fates, at least one of them has to be present for dislocation to the cytosol.

Summarizing the current evidence, one can draw the conlusion that the energy-dependence of peroxisomal protein import is caused by the cycle of the PTS-receptors. Retrotranslocation of the receptors is the energy-, and thus most likely the rate-limiting step of matrix-protein import. This energy-dependence can be separated into two groups of enzymatic activities: One the one hand, receptor ubiquitination by Pex4p, as ubiquitin has to be activated by E1 before Pex4p can be charged, and on the other hand, ATP hydrolysis in the conserved AAA-domain of Pex1p and Pex6p in order to pull the primed Pex5p out of the membrane.

A model is emerging in which the previously disparate roles of Pex4p and the AAA peroxins are combined in a concerted reaction sequence. For future research, it will be a challenge to elucidate how ATP-dependent receptor dislocation is mechanstically linked to import of folded proteins.

VII. References

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Figure Legends

Fig. 1: PTS-receptor cycle during peroxisomal matrix protein import.

The peroxisomal matrix protein import receptor Pex5p recognizes cargo proteins harboring the peroxisomal targeting signal type 1 (PTS1). Cargo proteins containing the PTS2-sequence bind to the PTS2-receptor Pex7p, which is associated with the co-receptors Pex18p and Pex21p in *S. cerevisiae*. The receptor/cargo complexes reach the peroxisomal membrane and associate with the docking complex, consisting of the integral membrane protein Pex13p as well as the peripheral Pex14p and Pex17p. The PTS-receptors reach the peroxisomal lumen, release their cargo and are dislocated back to the cytosol. The RING-finger-motif containing peroxins Pex2p, Pex10p and Pex12p form a complex that is connected to the docking-factors via Pex8p. The RING-finger proteins are supposed to be involved in the process of receptor

dislocation to the cytosol. Pex10p is linked to the ubiquitin-conjugating enzyme Pex4p, which is anchored to the peroxisomal membrane by Pex22p. The AAA-proteins Pex1p and Pex6p are involved in PTS-receptor release. They interact with each other and are attached to the peroxisomal membrane via Pex15p.

Fig. 2: Domain structure of AAA protein family members.

Pex1p and Pex6p as well as Sec18p and Cdc48p are members of the AAA-family of ATPases. These AAA-proteins share a common structural organization as they exhibit an amino-terminal domain ("N") and two AAA-ATPase domains ("D1","D2"). The AAA-domains contain an ATP-binding site ("Walker A") and a site for ATP-hydrolysis ("Waker B"). Depending on the degree of conservation of the AAA-domains, these AAA-ATPases can be devided into three groups. Sec18p is an example for type I with the first AAA domain ("D1") being better conserved than the second one ("D2"). Pex1p and Pex6p belong to the second type as their second domain is the one that is highly conserved. It is interesting to note, that Pex6p has no ATP-hydrolysis site in its first AAA-cassette. Cdc48p stands for type III, where both domains are well conserved. In many cases, the N-domain of the AAA-proteins proved to be capable to interact with adaptor proteins.

Fig. 3: Structure of the N-terminal domain (amino acids 3 to 180) of the AAA protein PEX1 from mouse

The polypeptide is folded into two globular subdomains. The N-terminal lobe folds as double-psi-barrel and the C-terminal loop as a beta-barrel. In spite of the low similarity (less than ten percent) with other N-domains of AAA-proteins, the overall structure is remarkably similar to the N-domains of Cdc48p/p97 or NSF, suggesting that these structures have a common function, e.g. in substrate binding. Image was generated form Protein Data Bank (PDB) structure 1WLF using Kinemage (Duke University, USA).

Fig. 4: Similarities between PTS-receptor recycling during peroxisomal matrix protein import and ERAD. The schematic representation of the Pex5-recycling pathway (left) and ERAD (right) displays similarities in terms of protein composition. Both contain integral RING-finger proteins that are proven E3 enzymes (Doa10p, Hrd1p) or have putative E3 activity (Pex2p, Pex10p, Pex12p). The peroxisomal E2 enzyme Pex4p is anchored via Pex22p, whereas Ubc7p is attached to the ER-membrane through Cue1p. Additionally, Ubc7p interacts with the membrane integrated Ubc6p. The members of the Ubc4p/Ubc5pUbc1p-family participate in both pathways, which also is the case for Uba1p, which charges the E2 enzymes with activated ubiquitin. Each system includes membrane anchored AAA-complexes. The AAA-ATPase Cdc48p interacts with the integral ER-membrane protein Der1p, whereas the AAA peroxin Pex6p binds to Pex15p in *S. cerevisiae* or Pex26p in human cells. Additionally, Pex6p forms a complex with the second AAA peroxin, Pex1p. Both AAA peroxins are required for dislocation of the PTS1-receptor Pex5 back to the cytosol. This is reminiscent of the function of Cdc48p, which translocates polyubiquitinated proteins from the ER-lumen to the cytosol for proteasomal disposal. This part of the pathway is branched in peroxisomes. While polyubiquitinated receptors are supposed to be degraded by the proteasome, monoubiquitination may function as signal for Pex5p recycling in order to facilitate another round of matrix protein import.

Fig. 5: Model for Pex5p ubiquitination and retrotranslocation. The cycling PTS1-receptor Pex5p has to be exported back to the cytosol after release of the cargo protein into the peroxisomal lumen. At this point of its cycle, Pex5p behaves like an integral membrane protein. Presumably three enzymatic activities, localized at the peroxisomal membrane, are

required for Pex5p release: (1) ubiquitin-conjugation, (2) ubiquitin-protein isopeptide ligation and (3) ATPase activity. Members of the Ubc4p/Ubc5p/Ubc1p-family function in polyubiquitination of Pex5p. This process requires the RING-finger peroxins Pex2p, Pex10p and Pex12p as well which may function as E3-enzymes. The polyubiquitinated forms of Pex5p can be recognized by the AAA peroxins Pex1p and Pex6p that are anchored to the peroxisomal membrane via Pex15p. PolyUb-Pex5p is extracted from the membrane in an AAA-peroxin and ATP-dependent manner and is degraded by the 26S proteasome. Pex5p destined for recycling is supposed to be modified by monoubiquitination. This process also requires the RING-finger peroxins and is proposed to be mediated directly by the peroxisomal Ubc Pex4p. Monoubiquitinated Pex5p is supposed to be dislocated by the AAA peroxins, deubiquitinated and thereby made available for further rounds of matrix protein import.

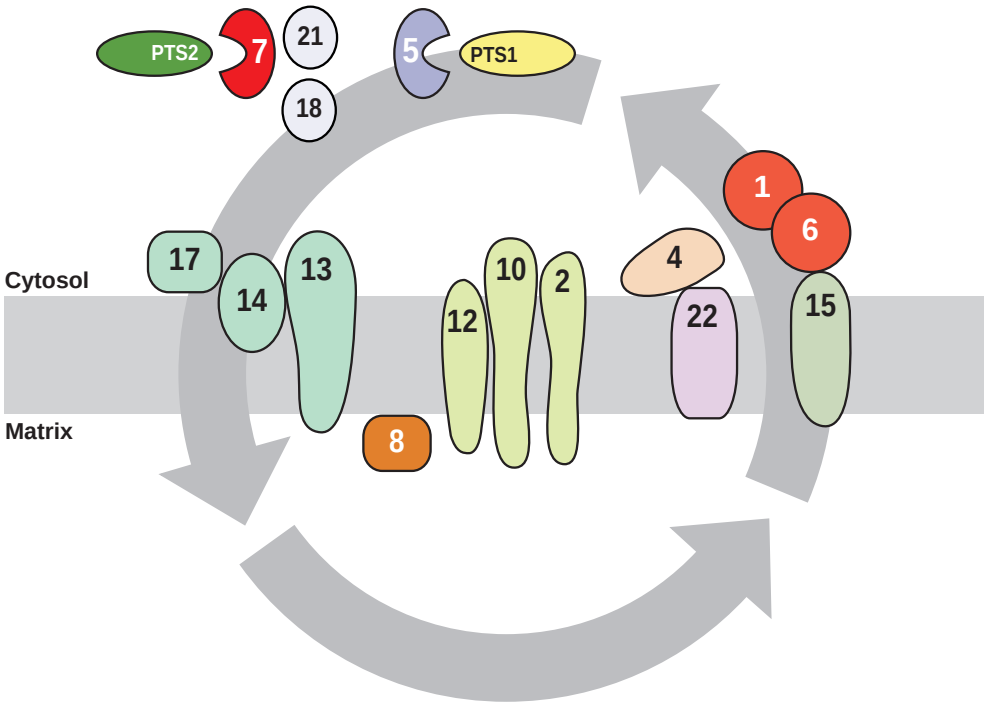

Fig. 1
Platta et al.

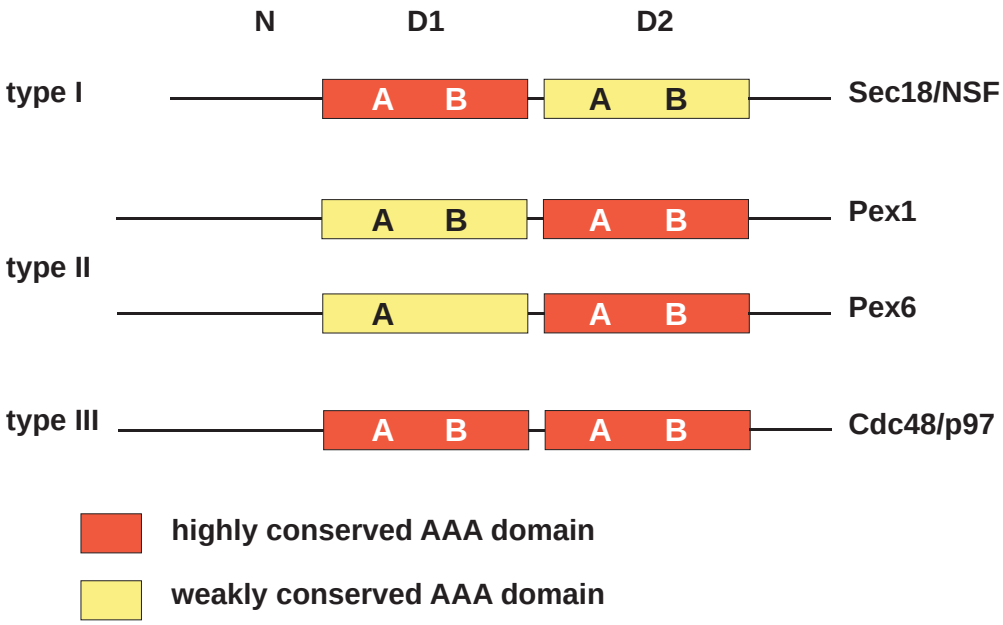

Fig. 2
Platta et al.

Fig. 3
Platta et al

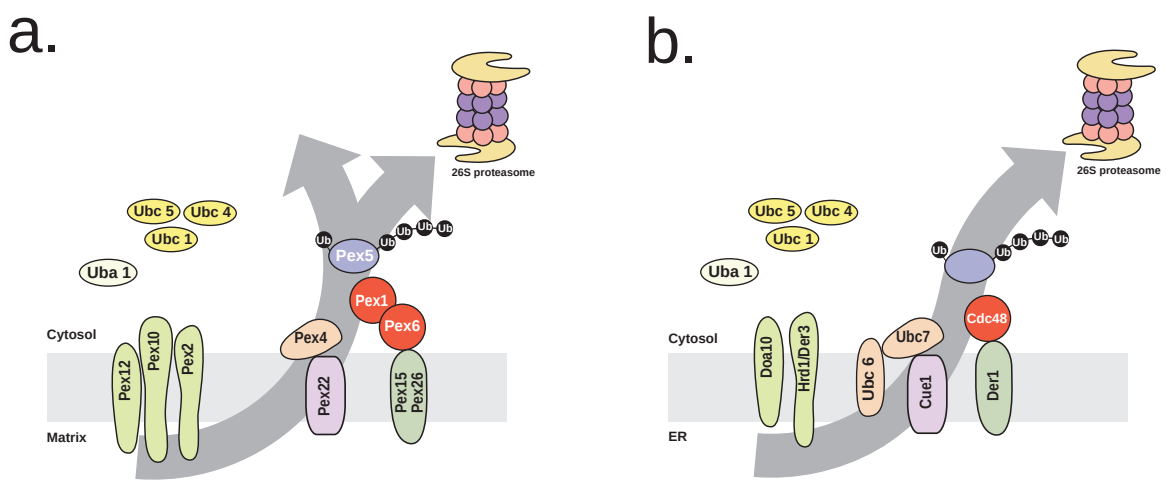

Fig. 4

Platta et al.

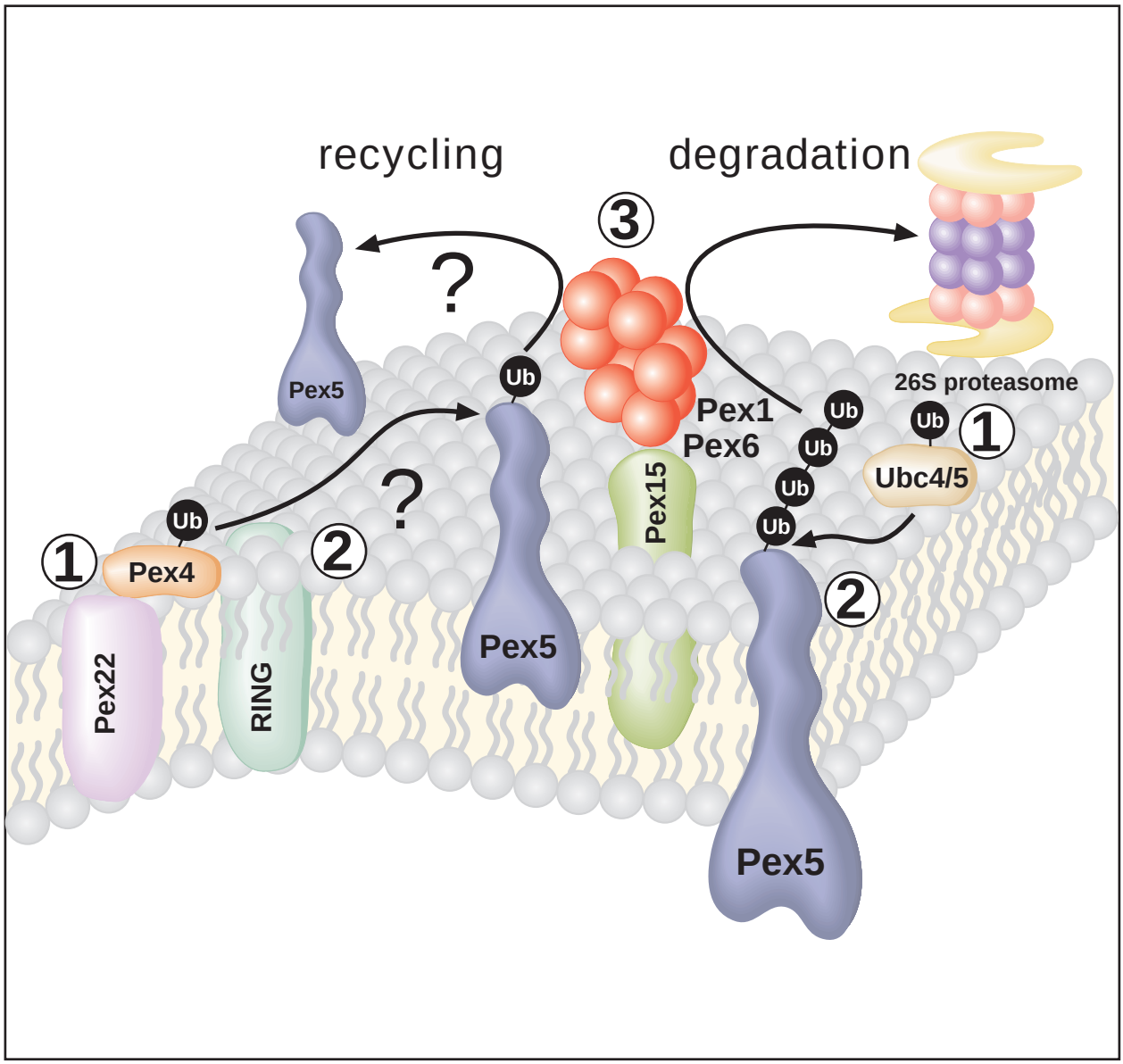

Fig. 5

Platta et al.

3. DISKUSSION

3.1 Eingliederung der Thematik in den Kontext peroxsiomaler Forschung

Das grundlegende Prinzip zur Aufrechterhaltung von biochemischen Gleichgewichten zwischen verschiedenen Stoffwechselprozessen eukaryontischer Zellen ist die Kompartimentierung. Die unterschiedlichen Organellen weisen jeweils eine charakteristische Zusammensetzung an Proteinen auf, welche für die jeweils intern ablaufenden Stoffwechselwege notwendig sind. Die meisten in Organellen vorhandenen Proteine sind kernkodiert, so dass sie nach der Translation an freien zytoplasmatischen Ribosomen anhand ihrer Signalsequenz spezifisch in die Zielkompartimente transportiert werden müssen (Blobel, 1979). Die Kompartimente, in welche Proteine transloziert werden, weisen spezifische Importmaschinerien auf. Bei den Peroxisomen, die als letzte der klassischen Kompartimente der eukaryontischen Zelle identifiziert worden sind (Rhodin, 1954, De Duve & Baudhuin, 1966), begann sich erst im Laufe des letzten Jahrzehnts ein kohärentes Bild über die Bestandteile und den Aufbau der peroxisomalen Importmaschinerie zu entwickeln (Lazarow, 2003). Die zentrale Position im Matrixproteinimport nehmen die PTS-Rezeptoren als Brücke zwischen der Entstehung der Proteine im Zytosol und deren Destination im Peroxisom ein. Ein Charakteristikum der PTS-Rezeptoren besteht darin, dass sie den Import von gefalteten, oligomeren Proteinen ermöglichen und zwischen dem Zytosol und dem Lumen des Peroxisoms pendeln. Dieses *extended shuttle* Modell zum Matrixproteinimport in Peroxisomen ist konzeptionell in vier Teilschritte untergliedert:

1. Erkennung der neusynthetisierten Matrixproteine durch lösliche Rezeptoren im Zytoplasma
2. Anbindung des Rezeptor-Matrixprotein-Komplexes an der peroxisomalen Membran
3. Translokation des Komplexes über die Membran und Freisetzung des Matrixproteins in das Peroxisom
4. Freisetzung des Rezeptors in das Zytoplasma.

Neben den hauptsächlich zytoplasmatisch vorkommenden PTS1- und PTS2-Rezeptoren Pex5p und Pex7p sind die meisten der für diese Prozesse notwendigen Peroxine an der peroxisomalen Membran lokalisiert. Eine funktionelle Zuordnung dieser Proteine zu den einzelnen Schritten des Rezeptorzyklus beruht fast ausschließlich auf Interaktionsstudien (Terlecky & Fransen, 2000), die zwar fundamentale Aussagen über die Bestandteile und

Assemblierung der Membrankomplexe lieferten, die aber in Bezug auf mechanistische Aspekte fast ausschließlich nur hypothetische Aussagen zu treffen in der Lage sind (Collins *et al.*, 2000).

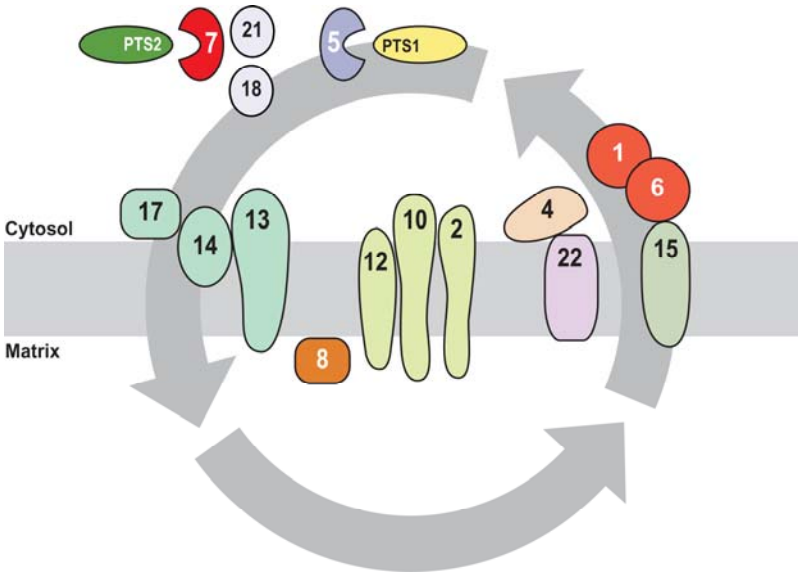

Abb.3.1 Zyklus der PTS-Rezeptoren während des peroxisomalen Matrixproteinimports. Das hier schematisch präsentierte *extended shuttle* Modell zum Matrixproteinimport in Peroxisomen ist konzeptionell in vier Teilschritte untergliedert: (1) Erkennung der neusynthetisierten Matrixproteine durch lösliche Rezeptoren im Zytoplasma, (2) Anbindung des Rezeptor-Matrixprotein-Komplexes an der peroxisomalen Membran, (3) Translokation des Komplexes über die Membran und Freisetzung des Matrixproteins in das Peroxisom, (4) Freisetzung des Rezeptors in das Zytoplasma (aus Platta *et al*., 2006b, verändert).

In den letzen Jahren stand die Identifizierung und Charakterisierung peroxisomaler Proteine im Mittelpunkt der Untersuchungen zur Biogenese von Peroxisomen. Nach der Identifizierung von bislang 32 PEX-Genen, die für diesen Prozess verantwortlich sind (zur Übersicht Baker & Sparkes, 2005), konzentriert sich die Forschung nun darauf, mehr über die Funktion und Regulation der einzelnen Peroxine im Verlauf der peroxisomalen Biogenese zu erfahren.

Das in dieser Arbeit dokumentierte Projekt sollte erste detaillierte Erkenntnisse über die Regulation sowie den molekularen Mechanismus bezüglich des Exports des PTS1-Rezeptors vom peroxisomalen Kompartiment zurück ins Zytosol ermöglichen. Die erzielten Ergebnisse lassen sich vier zentralen Fragestellungen zuordnen: (*i*) *in vivo* Analyse der Dynamik des PTS1-Rezeptors, (*ii*) Beschreibung und Charakterisierung der Polyubiquitinylierung und (*iii*)

Monoubiquitinylierung von Pex5p, sowie (*iv*) die Etablierung eines zellfreien *in vitro* Systems für den PTS1-Rezeptor, um in definierten experimentellen Bedingungen die mechanistische Bedeutung verschiedener Parameter in Bezug auf Anbindung an und vor allem die Freisetzung von der peroxisomalen Membran systematische testen zu können.

3.2 In vivo Analyse der Dynamik des PTS1-Rezeptors Pex5p

Der peroxisomale Matrixproteinimport wird durch mobile Rezeptoren und eine membranständige Importmaschinerie ermöglicht. Diese membranständige Maschinerie läßt sich mit Hilfe von direkten Interaktionsstudien zwischen einzelnen Proteinen sowie durch Isolierung von Multiproteinkomplexen in verschiedene Substrukturen unterteilen. Diese Beschreibung der sukzessiv identifizierten Komplexe erfolgte bisher in separaten Abhandlungen. Zu Beginn dieser Arbeit sollten daher zunächst weitere *in vivo* Hinweise für die postulierten Reaktionsfolge von Pex5p in den peroxisomalen Membrankomplexen im direkten Vergleich aller bekannten Komponenten gesammelt werden. Der experimentelle Ansatz bestand darin, die subzelluläre Lokalisation von Pex5p in verschiedenen Hefe-Mutanten, die in der Ausbildung definierter Membrankomplexe gestört sind, mittels Sedimentationsanalyse zu bestimmen.

Die Peroxine Pex13p, Pex14p und Pex17p werden in *Saccharomyces cerevisiae* als sogenannter „Docking-Komplex zusammengefaßt, welcher der stabilen Anbindung des Rezeptor-Kargo-Komplexes an die peroxisomale Membran dient und essentiell für den Proteinimport ist (zur Übersicht Eckert & Erdmann, 2003). Die im Rahmen dieser Arbeit durchgeführten vergleichenden Sedimentationsanalysen zur subzellularen Lokalisation des PTS1-Rezeptors Pex5p bestätigen die Funktion von Pex13p und Pex14p als „Docking“-Komponenten für Pex5p, da bei der Doppeldeletion fast das gesamte Pex5p im löslichen Überstand verbleibt (Platta *et al.*, 2005, Fig.1a). Während die Deletion von Pex8p, welche den „Docking“-Komplex vom RING-Finger-Komplex entkoppelt (Agne *et al.*, 2003), oder von Pex12p, dem Stabilisator des RING-Finger –Komplexes (Zhang *et al.*, 2006), zu einer Wildtyp-ähnlichen Verteilung von Pex5p zwischen Zytosol und Membran führte, lag der Großteil des PTS1-Rezeptors in Deletionsstämmen des AAA-Komplexes (*pex1*Δ, *pex6*Δ, *pex15*Δ) partikulär vor. Ein vergleichbares Ergebnis wurde in den *pex4*Δ und *pex22*Δ-Stämmen erzielt (Platta, unveröffentlicht). Somit erscheint die Lokalisation von Pex5p abhängig von der korrekten Assemblierung verschiedener peroxisomaler Membrankomplexe zu sein. Dies lässt darauf schließen, dass ein definiertes Angebot an Bindestellen für Faktoren der Membran in Abhängigkeit der einzelnen Importkomplexe besteht. Wertet man die Menge

an partikulärem Pex5p als Basis für eine Epistasis, steht zu Beginn der „Docking"-Komplex. Eine Verhinderung der Anbindung von Pex5p an die peroxisomale Membran wurde für *S. cerevisiae* schon in Sedimentationsanalysen in *pex14*Δ Zellen festgestellt (Albertini *et al.*, 1997). Dass die Membrananbindung durch das Fehlen von Pex8p nicht vermindert wird, kann ebenfalls anhand von publizierten Daten unterstützt werden (Rehling *et al.*, 2000). Neuartig ist jedoch die Beschreibung einer Akkumulation des PTS1-Rezeptors an der peroxisomalen Membran, wenn der AAA-Komplex (Platta *et al.*, 2005-Fig.1a) oder der Pex4p/Pex22p-Komplex (Platta, unveröffentlicht) nicht assembliert werden kann. Um genauer zu untersuchen, ob es sich bei diesem Phänomen um einen rein strukturellen Effekt, bedingt durch das Fehlen von Strukturkomponenten, handelt, oder aber ein funktioneller Zusammenhang zwischen den AAA-Peroxinen und dem Pex5p-Zyklus erstellt werden kann, wurden Punktmutanten von Pex1p und Pex6p getestet (Platta *et al.*, 2005-Fig.2e).

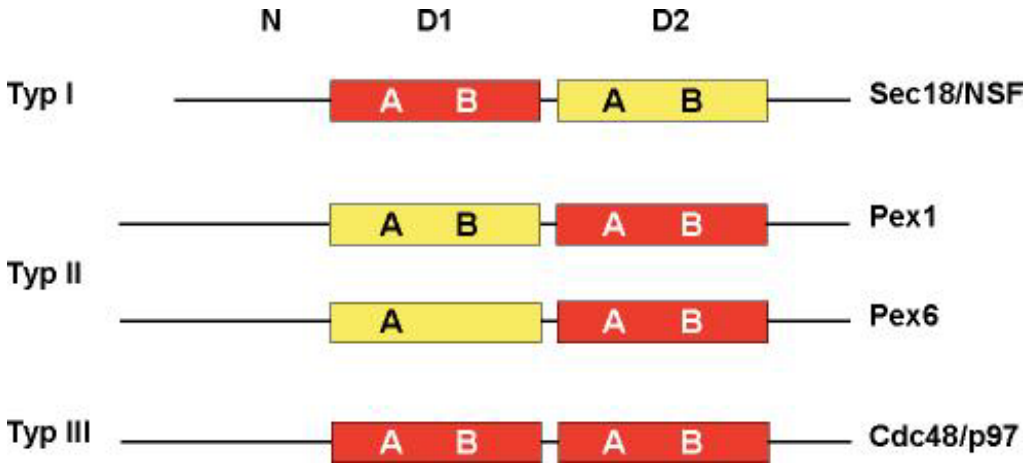

Abb.3.2 Strukturelle Organisation der Domänen von Pex1p und Pex6p. AAA-Proteine weisen im allgemeinen einen modularen Aufbau auf, wobei eine amino-terminale Domäne (N), die vor allem für Interaktionen mit Adaptor-Proteinen genutzt wird, einer oder mehrerer AAA-Domänen gegenüber gestellt wird. Wenn zwei AAA-Domänen vorhanden sind, kann entweder die erste (D1) gut konserviert sein (Typ I; z.B. NSF), die zweite Domäne (D2) kann gut konserviert sein (Typ II; z.B. Pex1p, Pex6p) oder aber beide Domänen können gut konserviert vorliegen (Typ III; z.B. Cdc48p). Jede Domäne beinhaltet prinzipiell ein ATP-Bindungs-Motiv (A) und ein ATP-Hydrolyse-Motiv (B). Interessanterweise besitzt Pex6p in der ersten Domäne nur ein ATP-BIndungsmotiv. (aus Platta *et al.*, 2006b, verändert).

Anhand von Komplexisolierungen mit Pex15p-TEV-ProtA konnte demonstriert werden, dass die Punktmutanten prinzipiell immer noch in der Lage sind, einen über Pex15p verankerten peroxisomalen Komplex zu bilden (Platta, unveröffentlicht). Die Sedimentationsanalyse zeigte auf, dass die Mutationen in den ATP-Bindungs- (Pex1p(K467E), Pex6p(K489A)) und ATP-Hydrolyse-Motiven (Pex1p(D525Q)) der ersten, weniger konservierten Domänen von Pex1p wie auch von Pex6p keinen signifikanten Einfluß auf die duale Verteilung von Pex5p zwischen löslicher und partikulärer Fraktion auszuüben scheinen. Dagegen resultiert die Mutation in den ATP-Bindungs- (Pex1p(K744E), Pex6p(K778A)) und ATP-Hydrolyse-Motiven (Pex1p(D797Q), Pex6p(D831Q)) der zweiten, hoch konservierten Domänen in einem ähnlichen Verteilungsmuster, wie die jeweiligen Deletionsstämme. Pex5p akkumuliert somit an der peroxisomalen Membran nicht nur, wenn die AAA-Peroxine fehlen, sondern ebenfalls, in dem Fall, dass sie zwar assembliert sind, jedoch die ATPase-Aktivität der zweiten AAA-Domäne inaktiv ist. Diese Befunde deuten auf eine unmittelbare Involvierung der genannten Proteine in den Rezeptorzyklus von Pex5p hin. Bisher hatte man allgemein die AAA-Peroxine aufgrund der vorhandenen Hinweise auf eine Funktion bei der Vesikelfusion (Faber *et al.*, 1998; Titorenko *et al.*, 2000; Titorenko & Rachubinski, 2001) begonnen separat von der eigentlichen Importmaschinerie anzusiedeln. Ähnliches galt für Pex4p, für das zumindest über das „SplitUbiquitin“-Interaktionssystem eine räumliche Nahe zu Pex10p festgestellt worden ist (Eckert & Johnsson, 2003), welches aber ansonsten verwaist und ohne bekannte Funktion oder gesicherte Interaktion mit dem Importomer blieb. Erst in einer aktuellen Publikation konnte über die Isolierung peroxisomaler Membrankomplexe eine strukturelle Verbindung des AAA-Komplexes mit dem Importomer nachgewiesen werden (Rosenkranz *et al.*, 2006). Die Akkumulation von Pex5p an der Membran kann somit als Hinweis auf eine funktionelle Bedeutung dieser Proteine bei dem Export-Schritt des Rezeptors gewertet werden.

Diese bisher besprochenen Daten unterstützen die ursprüngliche Vorstellung, dass es sich bei den AAA-Peroxinen wie auch bei Pex4p bzw. Pex22p um Peroxine handelt, denen eine späte Funktion im peroxisomalen Matrixproteinimport – und somit wahrscheinlich beim Recycling des Rezeptors - zukommt. Diese Vorstellung begründete sich vor allem darauf, dass in diesen Stämmen noch ein minimaler Restimport von Matrixproteinen detektierbar ist (Spong & Subramani, 1993; Heyman *et al.*, 1994; Koller *et al.*, 1999; Collins *et al.*, 2000). Die Kombination der erzielten Ergebnisse zur Rezeptor-Akkumulation in AAA-Mutanten mit den publizierten Daten zum Restimport von Matrixproteinen kann zu einem Arbeitsmodell vereint werden, nach dem Pex5p anbinden kann, ein Teil auch von Kargo zu dissoziieren in der Lage

ist, aber nicht mehr recycelt werden kann, so dass der Rezeptor akkumuliert und der Import versiegt. Dieser funktionelle Zusammenhang zwischen den „späten Peroxinen“ und dem Export von Pex5p, der zum Teil in diesen älteren Arbeiten schon diskutiert wird, sollte im Rahmen dieser Arbeit in einem neu etablierten *in vitro* System bestätigt und mechanistisch untersucht werden. Dies wird, in Kombination mit im Anschluß beschriebenen zusätzlichen Faktoren, an späterer Stelle dargelegt werden (3.4).

3.3 Identifizierung und Charakterisierung der Polyubiquitinylierung von Pex5p

Eine weitere Besonderheit der AAA- und Pex4p-Komplex Mutanten in *Saccharomyces cerevisiae* ist die Tatsache, dass, neben der Akkumulation an der Membran, in ihnen modifizierte Pex5p-Spezies detektierbar sind (Platta *et al.*, 2004-Fig.1; Kiel *et al.*, 2005a; Kragt *et al.*, 2005). Eine vergleichende Analyse der Zelllysate aller bekannten PEX-Deletions-Stämme von *S. cerevisiae* bestätigte, dass dies keinen allgemeinen *pex*-Phänotyp darstellt, sondern vielmehr ein Charakteristikum dieser fünf Stämme ist. Interessanterweise konnten die Mutanten anhand des spezifischen Musters dieser Modifikation in zwei Gruppen unterteilt werden. Die erste Gruppe beinhaltet *pex1*Δ, *pex6*Δ und *pex15*Δ, die alle jeweils drei zusätzliche Pex5p-Banden aufweisen, während *pex4*Δ und *pex22*Δ nur zwei dieser Modifikationsbanden zeigen, deren obere dabei der unteren des „*pex1*Δ-Musters“ entspricht. Diese Beobachtung spricht für die Annahme, dass alle fünf Peroxine einem gemeinsamen Funktionskontext angehören, der sich, wie die Muster zeigen, wiederum in zwei unterschiedliche Subkomplexe gliedert.

Aufgrund der Beobachtung, dass der PTS2-Co-Rezeptor Pex18p ubiquitinyliert wird (Purdue & Lazarow, 2001), wurde untersucht, ob die modifizierten Pex5p-Spezies durch Ubiquitinylierung entstehen. Mit dem klassischen Nachweis für Ubiquitinylierungsprodukte, nämlich dem Vergleich eines Stammes mit überexprimiertem Wildtyp Ubiquitin und einem *myc*Ubiquitin-überexprimierenden Stamm (Ellison & Hochstrasser, 1991), konnten zusätzliche Modifikationsbanden detektiert werden, die entsprechend der Anzahl an *myc*-Epitopen langsamer im SDS-Gel liefen (Platta *et al.*, 2004-Fig.2). Die nächste Frage bestand darin, welche Form der Ubiquitinylierung stattfindet, da dies eine grundsätzliche Aussage über die Funktion der vorliegenden Modifikation erlaubt. Je nachdem, ob einzelne Ubiquitin-Moleküle oder aber Ubiquitinketten, deren Monomere über verschiedene, charakteristische Lysin-Reste untereinender verknüpft sein können, an dem Zielprotein verankert vorliegen, wird das Zielprotein zu unterschiedlichen Orten gesteuert (Hicke *et al.*, 2005). Aufgrund der möglichen Verbindung, die zwischen der Ubiquitinylierung und der konstitutiven

Degradation von Pex18p besteht, wurde ein Inhibitor für K48-abhängige Polyubiquitinketten, Ubiquitin(K48R) (Hochstrasser *et al.*, 1991), überexprimiert (Platta *et al.*, 2004-Fig.6), da dieser Ketten-Typus in der Regel Proteine zur proteasomalen Degradation markiert. Dies resultierte in einer fast vollständigen Reduzierung der oberen Modifikationsbanden von Pex5p, was ein Indiz dafür ist, dass Pex5p in beiden Modifikationsgruppen mit K48-verknüpften Polyubiquitinketten modifiziert wird. Dass die oberen Modifikationsbanden nicht gänzlich ausgelöscht werden, läßt sich durch das Vorhandensein des endogenen Ubiquitins erklären.

Interessanterweise erfolgt die Polyubiquitinylierung nicht nur in den Deletionsstämmen, sondern auch in Stämmen deren jeweilige Proteine mittels gerichteter Mutagenese inaktiviert wurden, wie im Fall von Pex1p (Platta *et al.*, 2005-Fig. 2e; Kiel *et al.*, 2005a), Pex6p (Platta *et al.*, 2005-Fig. 2e) und Pex4p (Kiel *et al.*, 2005a). Somit geht die Ausbildung der Polyubiquitinkette an Pex5p mit der unter 3.2 diskutierten Akkumulation des Rezeptors an der Membran parallel einher.

Der in der beschriebenen Analyse des Modifikationstypus erhaltende Hinweis auf eine funktionelle Verbindung zum 26S Proteasom wurde mittels konditioneller Mutanten vertieft. *Cim5-1* ist eine temperatursensitive Mutante von Rpt1p, einer AAA-ATPase der 19S-Kappe des Proteasoms, welche an der Erkennung und Entfaltung der Substrate beteiligt ist (Ghislain *et al.*, 1993). Wachstum bei nicht-permissiver Temperatur führte zu einer Akkumulation von polyubiquitinyliertem Pex5p (Platta *et al.*, 2004-Fig.7), was einen proteasomalen Abbau belegt. Vergleichbare Ergebnisse wurden für die *cim3-1* (Rpt6p) Mutante berichtet (Kiel *et al.*, 2005a). Die proteasomale Degradation von Pex5p scheint, anders als im Fall von Pex18p, nicht konstitutiv zu sein, da Pex5p unter Wildtyp-Bedingungen ein sehr stabiles Protein mit einer Lebensdauer von über fünf Stunden ist (Kragt *et al.*, 2005). Vielmehr scheint es sich bei der Polyubiquitinylierung von Pex5p um ein Peroxisomen-spezifisches System zur Qualitätskontrolle der Rezeptoren zu handeln, das ein optimales Durchlaufen der Membrankomplexe und einen effizienten Export gewährleisten soll. Unter Bedingung, wenn das Recycling massiv gestört wird, wie bei der Deletion von Pex1p oder Pex4p, ist das System vom normalen Kreislauf entkoppelt und führt zu einer erhöhten Degradationsrate.

In Anlehnung an das Qualitätskontrollsystem für die Proteine des Endoplasmatischen Retikulums, ERAD (*endoplasmatic reticulum associated degradation*) (zur Übersicht Römisch, 2005), wurde kürzlich für dieses System, basierend auf den publizierten Daten in *S. cerevisiae* (Platta *et al.*, 2004; Kiel *et al.*, 2005a; Kragt *et al.*, 2005, Platta *et al.*, 2005), *H. polymorpha* (Kiel *et al.*, 2005b) und in *P. pastoris* (Leon *et al.*, 2006a), die Bezeichnung

RADAR (*receptor accumulation and degradation in absence of recycling*) vorgeschlagen (Leon *et al.*, 2006a; zur Übersicht Leon *et al.*, 2006b). Wie anhand der Versuche mit den *cim*-Mutannten gezeigt werden konnte, findet auch im Wildtyp die Degradation der Rezeptoren in begrenztem Umfang statt und wird in Mutanten der „späten Peroxine", einhergehend mit einer Akkumulation, verstärkt. Diese Resultate sind zum einen ein weiteres Indiz dafür, dass die AAA-Peroxine wie auch Pex4p und Pex22p eine Rolle bei der Freisetzung von Pex5p von der peroxisomalen Membran übernehmen. Zum anderen weist der Zusammenhang zwischen Akkumulation in diesen Mutanten sowie Polyubiquitinylierung und Degradation von Pex5p auf das Vorhandensein eines Systems zur Qualitätskontrolle der Rezeptoren hin, das dazu dient, dysfunktionales Pex5p, welches nicht mehr exportiert werden kann, zu entfernen.

Der nächste Schritt in der Charakterisierung der Polyubiquitinylierung von Pex5p bestand in der Aufdeckung der strukturellen Parameter, wie des Ortes, an dem die Ubiquitinylierung stattfindet, wie auch der beteiligten Enzyme und der modifizierten Lysine.
Um den Ort des Ubiquitiylierungsereignisses zu identifizieren, wurden zunächst Sedimentationsanalysen von *pex1*Δ, *pex4*Δ und *pex1*Δ*pex4*Δ Zelllysaten durchgeführt (Platta *et al.*, 2004-Fig.3a; Kiel *et al.*, 2005). Die polyubiquitinylierten Formen von Pex5p waren ausschließlich im Membransediment zu finden, was auf eine Modifikation an der peroxisomalen Membran hindeutete. Um spezifischere Aussagen treffen zu können, wurden Hefemutanten erstellt, die sowohl eine Deletion in PEX1 oder PEX4 aufweisen, als auch jeweils zusätzlich in einem weiteren Peroxin (Platta *et al.*, 2004-Fig.3b; Kiel *et al.*, 2005a). Anhand dieser Epistasis, wurde deutlich, dass Pex5p nach dem „Docking"-Schritt und zwar an oder nach dem RING-Finger Komplex modifiziert wird. Die Kombination von *pex1*Δ oder *pex4*Δ mit einer RING-Finger Deletion (*pex2*Δ, *pex10*Δ, *pex12*Δ) oder *pex8*Δ unterdrückte die Ausbildung der Modifikation vollkommen. Ähnliches wurde für die Kombination mit *pex13*Δ und *pex14*Δ beobachtet. Die Doppeldeletion *pex1*Δ*pex4*Δ führte zu einer Verstärkung der Ubiquitinylierung und ergab ein aus beiden Ub-Mustern gemischtes Modifikationsbild. Polyubiquitinylierung tritt somit spät im Weg von Pex5p an der Membran auf. Die Abhängigkeit von den RING-Finger Peroxinen Pex2p, Pex10p, Pex12p eröffnet die Möglichkeit, dass es sich bei diesen Proteinen um Ubiquitin-Protein-Isopeptid Ligasen (E3-Enzyme) handeln könnte, da eine der beiden Hauptklassen der E3-Enzyme das RING-Finger Motiv besitzt (RING-E3) (zur Übersicht Borden, 2000). Die Abhängigkeit nicht nur von einem individuellen sondern von allen drei Proteinen läßt sich damit begründen, dass diese sich im Komplex untereinander stabilisieren (Zhang *et al.*, 2006).

Das vierte bekannte peroxisomale RING-Finger Protein ist Trim37. Es wurde bisher nur im Menschen gefunden und besitzt eine *in vitro* nachgewiesene E3-Ligase-Aktivität (Kallijärvi *et al.*, 2005). Deletion oder distinkte Mutationen rufen im Menschen das autosomal rezessive *Mulibrey Nanism* (*muscle-liver-brain-eye*) Syndrom hervor, eine Krankheit, die als letzte peroxisomale Krankheit identifiziert wurde (zur Übersicht Karlberg *et al.*, 2006). Trim37, dessen Substrate und Funktion unbekannt sind, ruft bei Funktionsverlust jedoch keine Biogenesedefekte hervor.

Somit bleiben die RING-Finger Peroxine Pex2p, Pex10p und Pex12p die interessantesten Kandidaten. Da die RING-Finger Peroxine jedoch Teil der membrangebundenen Importkaskade sind, ist es nicht möglich durch ihre einfache Deletion die putative E3 Funktion nachzuweisen, da zum Beispiel die Deletion in dem vorgeschalteten PEX14 ebenfalls die Ubiquitinylierung verhindert. Daher werden zur Zeit Anstrengungen unternommen, die E3-Aktivität *in vitro* mit rekombinanten, aus *E. coli* aufgereinigten Proteinen nachzuweisen. Da die Volllängenkonstrukte dieser Membranproteine nicht löslich sind, werden die Experimente mit der jeweiligen RING-Domäne (Pex2p(215-271), Pex10p(238-337), Pex12p(293-399)) durchgeführt. Die Etablierung von Protokollen zur Autoubiquitinylierungsreaktion, Ubiquitinyliering eines S-Peptides als künstliches Substrat, sowie zur *in vitro* Ubiquitinylierung von rekombinantem Pex18p-His_6 sowie His_6-Pex5p und Pex5p-His_6 sind diesbezüglich als Zielsetzungen zu nennen..

Während somit die Funktion der RING-Finger Peroxine als trimerer E3-Ligase-Komplex noch nicht als zweifelsfrei bewiesen angesehen werden muss, konnten jedoch die Ubiquitin-konjugierenden Enzyme der Polyubiquitinylierung von Pex5p identifiziert werden. In einer systematischen Analyse wurden Doppeldeletionsmutanten von PEX1 bzw. PEX4 mit der Deletion eines Ubiquitin-konjugierenden Enzyms (E2-Enzym) kombiniert. Die Analyse ergab, dass in Kombination mit *ubc4*Δ die Polyubiquitinkette bei *pex1*Δ wie auch *pex4*Δ drastisch reduziert wurde (Platta *et al.*, 2004-Fig.4; Kiel *et al.*, 2005; Kragt *et al.*, 2005). Bis auf die Mutante *pex1*Δ *pex4*Δ, die, wie schon beschrieben, eine Intensivierung der Ubiquitinylierung aufweist, zeigten die andern Stämme keinen Effekt. Die restliche Ubiquitinylierung wird von Ubc5p und Ubc1p durchgeführt. Diese drei E2-Enzyme bilden eine kleine Familie und können sich in bestimmten Zusammenhängen zumindest partiell ersetzen (Seufert *et al.*, 1990; Seufert & Jentsch; 1990). Dies scheint auch im Fall der Polyubiquitinylierung von Pex5p zuzutreffen, da es in *pex1*Δ bzw. *pex4*Δ Stämmen, die mit der Doppledeletionen *ubc4*Δ *ubc5*Δ (Platta *et al.*, 2004) oder *ubc4*Δ *ubc1*Δ (Kragt *et al.*, 2005) kombiniert wurden, zu einer weiteren Abschwächung der Polyubiquitinylierungsbanden

kommt. Die individuelle Funktion von Ubc4p ist nicht essentiell für die peroxisomale Biogenese, da der Stamm *ubc4*Δ immer noch - ähnlich dem Wildtyp - in der Lage ist auf Ölsäure-Platten zu wachsen (Platta *et al.*, 2004-Fig.5a; Kiel *et al.*, 2005; Kragt *et al.*, 2005). Weder die drei einzelnen Deletionsstämme, noch die Doppeldeletion *ubc4*Δ *ubc1*Δ (Kragt *et al.*, 2005) wiesen einen wahrnehmbaren Wachstumsdefekt auf. Die gleichzeitige Deletion von *ubc4*Δ und *ubc5*Δ (Platta *et al.*, 2004) hatte keinen Einfluss auf das Wachstum auf Glukose-Platten, resultierte jedoch unter Peroxisomen-selektiven Bedingungen in einen partiellen Wachstumsdefekt, der sich durch langsameres Wachstum auf Oleat-Platten (Fig.5a), partieller zytosolischer Mislokalisation der Katalaseaktivität (Fig.5b) sowie partieller zytosolische Mislokalisation von GFP-SKL bei der *in vivo* Fluoreszenzmikroskopie manifestiert (Fig.5c).
Die Deletion aller drei E2-Enzyme ist letal für die Zelle (Seufert & Jentsch, 1990). Der partielle Wachstumsdefekt unter selektiven Bedingungen kann damit erklärt werden, dass die Ubc4p/Ubc5p/Ubc1p-katalysierte Polyubiquitinylierung von Pex5p eine regulative Aufgabe während der Biogenese innehat. So könnte die Menge an benötigtem Rezeptor den metabolischen Umständen angepaßt werden. Zudem können nicht mehr funktionelle Rezeptor-Moleküle, welche die Vorgänge an der Membran stören würden, auf diesem Weg entsorgt werden.
Um die Funktion der Polyubiquitinylierung von der Pex5p-Seite aus untersuchen zu können, wurden die modifizierten Lysin-Reste mittels gerichteter Mutagenese identifiziert.
Der Bereich der Polyubiquitinylierung wurde zunächst durch Analyse von Pex5p-Verkürzungskonstrukten im *pex1*Δ*pex5*Δ bzw. *pex4*Δ*pex5*Δ Hintergrund auf die Aminosäuren 17-313 eingegrenzt, wonach die darin vorhandenen 16 Lysine mittels gerichteter Mutagenese einzeln durch Arginin ersetzt wurden. Erst der gleichzeitige Austausch von K18 und K24 verhinderte die Polyubiquitinylierung (Platta *et al.*, 2006c-Fig.2a). Das K18 ist in *Sc*Pex5p das erste Lysin im N-Terminus und zudem hoch konserviert (Abb.3.3). Das K24 als das benachbart liegende, weniger konservierte Lysin, kann in Pex5p(K18R) die Funktion des konservierten Lysins übernehmen. Für dieses Prinzip der Sicherstellung einer effizienten Modifizierung des Zielproteins durch redundante Lysin-Reste, existieren aus anderen Systemen ähnliche Beispiele (Baldi *et al.*, 1996). Dieser Mechanismus scheint jedoch in Bezug auf die PTS-Rezeptoren bisher spezifisch für *S. cerevisiae* zu sein, da bei Pex5p aus *Hansenula polymorpha* (Kiel *et al.*, 2005b) und dem PTS2-Ko-Rezeptor Pex20p aus *Pichia patoris* (Leon *et al.*, 2006a) die Mutation des konservierten Lysins ausreichend ist, um die Polyubiquitinylierung zu verhindern.

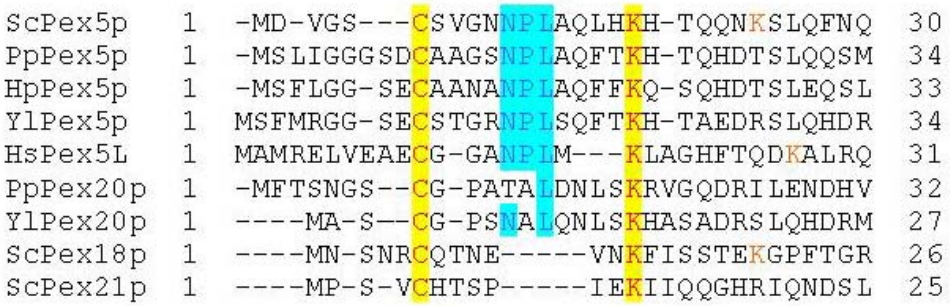

Abb. 3.3 Vergleich der Aminosäuresequenz der Amino-Termini verschiedener PTS-Rezeptoren. Die N-termini verschiedener PTS1-Rezeptoren und PTS2-Ko-Rezeptoren sind anhand ihrer Aminosäuresequenz verglichen worden. Farbig hervorgehoben sind das putative Export Signal Cys-X_n-Asn-(Ala/Gly)-(Leu/Ala) sowie der hoch konsrvierte Lysin-Rest, der in *Hp*Pex5p und *Pp*Pex20p die Polyubiquitinkette trägt, während bei *Sc*Pex5p zusätzlich noch das benachbarte Lysin involviert ist (rot). Dies läßt auf eine ähnliche Regulation für PTS1- und PTS2-Ko-Rezeptoren schließen. Pex7p (nicht gezeigt) besitzt keine vergleichbaren Motive.

Pex5p(K18R; K24R) komplementiert den PTS1-Import, was in der Fluoreszenzmikroskopie durch ein deutlich punktiertes Muster demonstriert wird. (Platta *et al.*, 2006c-Fig.1a). Die sensibleren Wachstumskinetiken in flüssigem Oleat-Medium weisen der Dopplepunktmutante ein etwas langsameres Wachstum als dem Wildtyp zu (Platta, unpubliziert).

Der vergleichsweise deutlicher hervortretende partielle Importdefekt in *ubc4*Δ*ubc*5Δ Zellen kann als ein akkumulierender Effekt interpretiert werden, da dort zusätzlich der konstitutive Degradationsprozess von Pex18p und Pex21p vermindert ablaufen dürfte und somit eine größere Anzahl an nicht funktioneller Rezeptoren die Kapazitäten an der Membran belasten sollte. Die korrespondierenden Mutationen in *H. polymorpha*, Pex5p(K21R), wie auch in *P. pastoris*, Pex20p(K19R), verhindern ebenfalls das Wachstum auf Oleat-Platten nicht (Kiel *et al.*, 2005b; Leon *et al.*, 2006a). Interessanterweise verhindert die N-terminale Fusion von drei *myc*-Epitopen ebenfalls die Ausbildung der Polyubiquitinkette (Platta *et al.*, 2006c-Fig.1c). Diese mögen die räumliche Zugänglichkeit zu K18 und K24 verhindern oder Bindestellen für Ubc4p oder das E3-Enzym maskieren.

Aufgrund der Beobachtung, dass die Stabilität von Pex18p in *S. cerevisiae* ebenfalls von Ubc4p und Ubc5p abhängig ist (Purdue & Lazarow, 2001) und dass in Pex20p aus *P. pastoris* der gleiche konservierte Lysin-Rest polyubiquitinyliert wird (Leon *et al.*, 2006a), ist

zumindest für die Ko-Rezeptoren des PTS2-Weges ein ähnlicher Mechanismus für Ubiquitinylierung und Degradation wie für Pex5p anzunehmen.

Die Effizienz der proteasomalen Degradation variiert deutlich zwischen verschiedenen Spezies. Während Pex5p in *S. cerevisiae* noch stark polyubiquitinyliert vorliegt, fällt die Konzentration an Pex5p in *H. polymorpha* (van der Klei *et al.*, 1998; Kiel *et al.*, 1999), *P. pastors* (Collins *et al.*, 2000), Pflanzen (Zolman & Bartel, 2004) und menschlichen Zelllinien (Dodt & Gould, 1996; Yahrhaus *et al.*, 1997) dramatisch ab.

Somit liefert die Beschreibung der Polyubiquitinylierung von Pex5p die lang gesuchte Erklärung für die niedrigen Konzentrationen des PTS1-Rezeptors in Patientenzellen der Komplementationsgruppen 1 (*PEX1*), 4 und 6 (*PEX6*) sowie 8 (*PEX26*).

3.4 Charakterisierung der Monoubiquitinylierung von Pex5p

Neben der beschriebenen Polyubiquitinylierung von Pex5p (Platta *et al.*, 2004), die in Mutanten der „späten Peroxine“ wie auch in proteasomalen Mutanten auftritt, kann der PTS1-Rezeptor in Wildtyp-Zellen monoubiquitinyliert werden (Kragt *et al.*, 2005). Monoubiqutinylierung von Pex5p ist eine transiente Modifikation, die nur durch Inhibierung der deubiquitinylierenden Enzyme sichtbar gemacht werden kann. Diese Inhibierung erfolgt mittels N-ethylmaleimid (NEM), welches Thiol-Gruppen alkyliert und somit die deubiquitinylierenden Enzyme, welche in *S. cerevisiae* Cystein-Proteasen sind, inhibiert.

Diese Modifikation ist unabhängig von Ubc4p und Ubc1p, benötigt jedoch die Anwesenheit der RING-Finger Peroxine und findet somit an der peroxisomalen Membran statt (Kragt *et al.*, 2005). Die Analyse der Monoubiquitinylierung von Pex5p wurde jedoch durch das Vorhandensein der polyubiquitinylierten Formen in den Deletionsstämmen der „späten Peroxine“ erschwert, so dass diese Charakterisierung mit *myc*Pex5p und Pex5p(K18R;K24R), die nicht mehr polyubiquitinyliert werden können, fortgesetzt werden sollte.

Die Behandlung der *myc*Pex5p bzw. Pex5p(K18R; K24R) enthaltenen Zelllysate mit NEM führte zur Detektion einer einzigen NEM-spezifischen Pex5p bei Ub und Ub(K48R) überexprimierenden Stämmen, während bei *myc*Ub Überexpression eine größere zusätzliche Bande zu erkennen war (Platta *et al.*, 2006c-Fig.1d). Das Experiment mit Ub(K48R) zeigte, dass es sich hierbei tatsächlich um Monoubiquitinylierung handelt. Kragt *et al.* (2005) gehen aufgrund der Größe ihrer Bande von zwei einzelnen Ubiquitin-Molekülen aus. Auch die im vorliegenden Experiment detektierbare Bande läuft bei ca. 90 kDa, was einer Höhe von di-MonoUb-Pex5p entspräche. Um die Frage nach der genauen Anzahl der einzelnen Ubiquitin-Moleküle experimentell beantworten zu können, wurden Versuche mit Stämmen, die

*myc*Ubiquitin überexprimieren durchgeführt (Platta *et al.*, 2006c-Fig.1d). In der Abbildung sind deutlich nur zwei Banden zu sehen, was zunächst auf das Vorhandensein nur eines einzelnen Ubiquitins hindeutet, welches vermutlich das Laufverhalten von monoUb-Pex5p verändert. Bei einer Modifikation mit zwei Ubiquitin-Molekülen wären drei Bande (Ub-Ub/*myc*Ub-Ub/*myc*Ub-*myc*Ub) zu erwarten gewesen. Somit ist von einer einfachen Monoubiquitinylierung auszugehen.

Um das für die Monoubiquitinylierung verantwortliche E2-Enzym zu identifizieren, wurde *myc*Pex5p in die entsprechenden Deletionsstämme transformiert und unter NEM-Bedingungen immunoprezipitiert (Platta *et al.*, 2006c-Fig.2a). Mit Ausnahme des *pex4*Δ Stammes konnte in allen getesteten *ubc*Δ Stämmen noch monoUb-Pex5p nachgewiesen werden, was als ein deutlicher Hinweis darauf gewertet werden kann, dass Pex4p das gesuchte Ubc darstellt. Nicht getestet wurden Ubc9p, da es nicht Ubiquitin, sondern Smt3p (SUMO) konjugiert. Ubc12p wurde ausgelassen, da es das ubiquitin-ähnliche RUB1 (NEDD 8) konjugiert. Einzig Ubc3p konnte nicht untersucht werden, da seine Deletion letal für die Zelle ist (zur Übersicht Weissman *et al.*, 2001). Auch wenn Ubc3p eine Funktion in der Monoubiquitinylierung von Pex5p in Wildtyp Zellen haben sollte, so spielt es definitiv keine Rolle in *pex4*Δ Zellen, da dort keine ubiquitinylierten Formen von *myc*Pex5p zu detektieren sind.

Zudem konnte gezeigt werden, dass die Aktivität und die peroxisomale Lokalisation für die Ausbildung der monoubiquitinylierten Form von *myc*Pex5p und Pex5p(K18R;K24R) notwendig waren, da keine Monoubiquitinylierung in einem Pex4p(C115S) oder *pex22*Δ Stamm zu detektieren war (Platta *et al.*, 2006c-Fig.2b + Fig.S1a). Die Monoubiquitinylierung von Pex5p erfolgt an oder nach den RING-Finger Peroxinen (Kragt *et al.*, 2005), jedoch vor den AAA-Peroxinen (Platta *et al.*, 2006c-Fig.2c + Fig.S1b). Diese Epistasis steht im Wiederspruch zu Daten aus *P. pastoris* (Collins *et al.*, 2000), die, aufgrund der Instabilität von Pex5p in PEX1- und PEX4-defizienten Stämmen zu dem Resultat kamen, dass die Funktion von Pex4p und Pex22p nach den AAA-Peroxinen erfolgt. Dies wurde damit begründet, dass die Kombination *pex1*Δ*pex4*Δ einen Abbau von Pex5p auf den Level von *pex1*Δ erkennen ließ. Kritisch betrachtet ist diese Folgerung jedoch nicht ganz zulässig, da Pex5p in *P. pastoris pex4*Δ Zellen so gut wie gar nicht mehr detektierbar war und somit ein eventuell auftretender Mischphänotyp unerkannt bliebe. In der Tat führt in *S. cerevisiae* diese Kombination nicht zu einem *pex1*Δ PolyUb-Muster, sondern zu einen Mischphänotyp beider Ketten-Muster (Platta *et al.*, 2004). Ist somit die Epistasis in *P. pastoris* in Bezug auf Pex4p kritisch zu sehen, sind beide Situationen wahrscheinlich ohnehin schwer oder gar nicht

vergleichbar, da die Degradation bzw. Polyubiquitinylierung ein pathologischer Marker darstellt, während die Monoubiquitinylierung unter Wildtyp Bedingungen vorherrscht.
Während mit Pex4p das E2-Enzym der Monoubiquitinylierung identifiziert werden konnte, bleibt der modifizierte Rest an Pex5p unbekannt. Die Analyse der 16 zuvor erstellten K zu R Mutanten hatte keinen Einfluss auf die Monoubiquitinylierung. Somit könnte auch hier die Existenz mehrerer redundanter Reste angenommen werden. Alternativ wäre eine Modifikation des konservierten Cysteinrestes (C6 in *Sc*Pex5p) oder aber der α-Aminogruppe der N-terminalen Aminosäure denkbar, da sich für beide Möglichkeiten in der Literatur Beispiele finden lassen (Ciechanover & Ben-Saadon, 2004; Cadwell & Coscoy, 2005). Deutlich wird jedoch, dass Mono- und Polyubiquitinylierung an verschiedenen Resten auf Pex5p erfolgen, da Pex5p(K18R; K24R) monoubiquitinyliert wird. Pex5p kann somit sowohl poly- als auch monoubiquitinyliert werden. Während die Polyubiquitinylierung in der Regel der Degradation im 26S Proteasom dient, hat die Monoubiquitinylierung oftmals regulative Funktion und verändert in den meisten Fällen die subzellulare Verteilung eines Proteins (Hicke *et al.*, 2005). Ob diese allgemeine Funktion auch bei der Monoubiquitinylierung von Pex5p zu tragen kommt, sollte im Anschluss in einem zellfreien *in vitro* System analysiert werden (3.5)

3.5 Etablierung eines zellfreien in vitro Systems für den PTS1-Rezeptor

Die in den Abschnitten 3.2, 3.3 und 3.4 diskutierten Daten zeigen deutlich einen Zusammenhang zwischen Akkumulation und Ubc4p-vermittelter Polyubiquitinylierung von Pex5p in Mutanten des AAA-Komplexes wie auch des Pex4p/Pex22p-Komplexes auf. Zudem konnte gezeigt werden, dass Pex5p in Wildtyp-Zellen von Pex4p monoubiquitinyliert wird.
Die Summe der präsentierten Evidenz stellt eine Verbindung von den AAA-ATPasen Pex1p und Pex6p, deren Membrananker Pex15p sowie des E2-Enzyms Pex4p, dessen Membrananker Pex22p sowie der Ubiquitinylierung von Pex5p zu dessen Dislokation her. Um die Funktion der genannten Faktoren im Kontext des Exportes von Pex5p in definierten Experimenten bestätigen zu können und zudem mechanistische Details der Dislokation zu untersuchen, wurde ein zellfreies *in vitro* System für *S. cerevisiae* erstellt.
Die Etablierung von *in vitro* Systemen für den peroxisomalen Matrixproteinimport in *S. cerevisiae* ist in der Vergangenheit dadurch erschwert worden, dass nicht zwischen Anbindungs- und Ablösungsreaktion des Rezeptors unterschieden werden konnte. Die verschiedenen Pex5p-Populationen konnten somit nicht getrennt werden Dies erschwerte die experimentelle Suche nach den benötigten Parametern zur Etablierung eines solchen Systems.

Die Entdeckung und Charakterisierung der Ubiquitinylierung von Pex5p erleichterte diesen Prozeß. Da sie spät im Pex5p Zyklus an der peroxisomalen Membran stattfindet, konnte sie als Marker benutzt werden, der spezifisch die Pex5p-Spezies kennzeichnete, welche schon den „Docking"- Komplex passiert und den RING-Finger-Komplex erreicht hat und zudem integral in der Membran inseriert.

Der erste konkreten *in vivo* Hinweis auf die Funktion der AAA-ATPasen Pex1p und Pex6p als Dislokasen für Pex5p wurde in Sedimentationsanalysen gewonnen (Platta *et al.*, 2005-Fig.1c). Während polyubiquitinyliertes Pex5p in einem *pex1*Δ Stamm ausschließlich im Membransediment zu finden war, konnte es in einer *cim5-1* Mutante auch zum Teil in der zytosolischen Phase detektiert werden. Die kombinierte Mutante *cim5-1/pex1*Δ wies wieder ausschließlich partikuläres PolyUb-Pex5p auf. Die Verteilung des löslichen PolyUb-Pex5p schien somit abhängig von Pex1p zu sein.

Um die mögliche Dislokase-Funktion der AAA-Peroxine genauer untersuchen zu können, wurde ein zellfreies *in vitro* System etabliert. Der *in vitro* Ansatz besteht in seiner einfachsten Form aus einem Pex5p oder PolyUb-Pex5p enthaltenden 100.000xg Membransediment, welches mit verschiedenen behandelten zytosolischen Überständen unterschiedlicher Stämme inkubiert und danach erneut durch Sedimentation getrennt wird. Danach kann die Verteilung von Pex5p bzw. PolyUb-Pex5p zwischen den beiden Fraktionen im Western Blot ermittelt werden (Platta *et al.*, 2005, Fig.2a + 2c). In weiteren Spezifizierungsschritten wurde der zytosolische Überstand durch den über Pex1p-TEV-ProtA affinitätschromatographisch isolierten zytosolischen Pex1p-Pex6p-Komplex substituiert (Platta *et al.*, 2005-Fig.3a bis c). Zusätzlich konnte das Membransediment durch intakte Wildtyp-Peroxisomen, die über einen Saccharose-Gradienten gereinigt worden waren, oder alternativ durch *pex1*Δ*pex6*Δ *ghosts*, die mittels Flotation gereinigt wurden, ersetzt werden (Platta *et al.*, 2005-Fig.3d + e). Letztendlich konnte der gesamte Zyklus von Pex5p *in vitro* rekonstituiert werden (Platta *et al.*, 2005-Fig.4).

Dieses System erlaubt die Variation einzelner Parameter und bietet somit die Möglichkeit definierte Fragestellungen zu untersuchen. Zunächst sollte die mögliche Energie-Abhängigkeit von Anbindung des Rezeptors bzw. dessen Ablösung untersucht werden

Der Freisetzung von Pex5p und PolyUb-Pex5p von der peroxisomalen Membran erwies sich als ATP-abhängig, während die Anbindung ATP-unabhängig erfolgte (Platta *et al.*, 2005-Fig.2a + 2e + Fig.4). Dies wurde schon in einem zellfreien System mit Peroxisomen aus Rattenleber gefunden (Gouveia *et al.*, 2003) und bestätigte die Funktionalität des Hefe-Systems. Während sich die Studien mit den Rattenleber Peroxisomen vornehmlich mit

energetischen, thermodynamischen und strukturellen Elementen bezüglich Pex5p selber auseinandersetzten (Gouveia *et al.*, 2003; Oliveira *et al.*, 2003; Costa-Rodrigues *et al.*, 2004) wurde das Hefe-System dazu genutzt, um die Funktionselemente der Exportmaschinerie zu identifizieren. Dabei wurde deutlich, dass die AAA-Peroxine Pex1p und Pex6p essentiell für die Dislokation von Pex5p als auch PolyUb-Pex5p sind (Platta *et al.*, 2005-Fig.2a + 2c). Die beiden AAA-Peroxine bilden einen ternären Komplex mit Pex15p und Pex5p an der Membran (Platta *et al.*, 2005-Fig.1b; Rosenkranz *et al.*, 2006). Sie sind nicht redundant, da sie auch bei Überexpression nicht das Fehlen des anderen zu kompensieren vermögen (Platta *et al.*, 2005-Fig.S1c). Die Analyse der ATPase-Mutanten von Pex1p und Pex6p ergab, dass beide AAA-Proteine sowohl die ATP-Bindung als auch ATP-Hydrolyse in ihrer jeweiligen konservierten AAA-Domäne benötigen, um ihre Funktion erfüllen zu können (Platta *et al.*, 2005-Fig.2f). Die mechanistische Konsequenz der Pex6p(K778E) und Pex6p(D831Q) Mutationen liegt wahrscheinlich in einer Blockierung der AAA-Pex5p-Komplex Disassemblierung. Basierend auf Quantifizierung von Zweihybrid-Interaktionstests wurde für die konservierte Domäne von Pex6p eine regulative Rolle bei der Disassemblierung des Pex6p-Pex15p Komplexes vorgeschlagen (Birschmann *et al.*, 2003). Dieser Befund steht in Analogie zu der Funktion der konservierten Domäne von Sec18p(NSF), welche ebenfalls zur Dissoziation der SNARE-Komplexe benötigt wird (May *et al.*, 2001). Ein anderer Hinweis, dass der Rezeptorzyklus von Pex5p und der ATPase-Zyklus von Pex6p an Pex15p konvergieren, ist die Beobachtung, dass neben Pex5p vor allem Pex1p und Pex6p in *PEX4*-defizienten Zellen spezifisch im Komplex mit Pex15p akkumulieren (Rosenkranz *et al.*, 2006). Des Weiteren können die AAA-Peroxine als Dislokasen angesprochen werden, da sie integrale Formen von Pex5p aus der Membran lösen und exportieren können (Platta *et al.*, 2005-Fig.2a + 2b + Fig.S1b). Als zusätzlicher essentieller Bestandteil der Dislokationsmaschinerie konnte der Membrananker Pex15p identifiziert werden (Platta *et al.*, 2005-Fig.2d). Diese Daten belegen eindrücklich die aufgrund der Akkumumulation (3.2) und Polyubiquitinylierung (3.3) von Pex5p in diesen Stämmen angenommene Funktion bei der Freisetzung des PTS1-Rezeptors.

Als weiterer Aspekt konnte festgestellt werden, dass die Voraussetzung für eine effiziente Rezeptor-Dislokation in einer stabilen Assoziation von Pex5p mit dem „Docking“-Komplex besteht, jedoch eine solche nicht essentiell zu sein scheint. Pex5p konnte während gekoppelter *in vitro* Anbindungs- und Export-Reaktionen in einem Pex13p/Pex14p-defizienten System weder spezifisch angebunden noch exportiert werden (Kerssen *et al.*, 2006-Fig.5b). Die Punktmutante Pex5p(W204A), die keine Interaktion mit Pex13p mehr im Zweihybrid System

zeigt (Bottger *et al.*, 2000; Schell-Steven *et al.*, 2005; Kerssen *et al.*, 2006-Fig.1), beeinträchtigte die *in vitro* Anbindung an die Membran nicht signifikant, sondern hatte ausschließlich einen deutlicheren Effekt auf den Export von Pex5p (Kerssen *et al.*, 2006-Fig.5a). Die Kombination von W204A mit der neu beschriebenen W261A Mutation in dem inversen *WxxxF*-Motiv von Pex5p für eine der Interaktionen zu Pex14p, führte zu einer dramatischen Instabilisierung der Assoziation von Pex5p mit dem Importomer, was durch Komplexisolierungen mit TEV-ProtA fusionierten Pex5p-Mutanten festgestellt worden war (Kerssen *et al.*, 2006-Fig.2a). Dieser Verlust des stöchiometrischen Verhältnisses zu den Bindepartnern reduzierte die Menge *in vitro* gebundenem Pex5p nicht signifikant, was mit einer intrinsischen Lipid-Bindefähigkeit von Pex5p erklärbar ist (Kerssen *et al.*, 2006). Der Export war jedoch drastisch reduziert (Kerssen *et al.*, 2006-Fig.5a), was die Notwendigkeit einer stabilen Assoziation mit dem Importomer verdeutlicht.

Die Entdeckung der AAA-Protein-abhängigen Dislokation von integralem Pex5p erlaubt verschiedene Vergleiche zu anderen Translokationssystemen.

Die proteinogene Zusammensetzung der peroxisomalen Matrixproteinimportmaschinerie ähnelt der des ERAD-Systems, welches fehlgefaltete Proteine aus dem ER ins Zytosol zur proteasomalen Degradation zurückführt (Abb. 3.4). In beiden Fällen stellt ein AAA-ATPase Komplex die „Motor-Komponenete“ dar. Wird das Pex1p/Pex6p-Heterohexamer über Pex15p (bzw. Pex26p in Mensch) an die Membran bebunden, assoziiert das Cdc48p-Hexamer über Der1p (bzw. Derlin-1/VIMP in Säugern) an die jeweilige Membran (Ye *et al.*, 2001; Jarosch *et al.*, 2002; Ye *et al.*, 2004; Lilley & Ploegh, 2004). Beide Systeme weisen je eines der beiden einzigen E2-Enzyme mit Membrananker – Pex4p/Pex22p bzw. Ubc7p/Cue1p (Vashist & Ng, 2004) – auf. Zudem existieren am Endoplasmatischen Reticulum mit Hrd1p und Doa10p zentrale RING-E3-Komplexe (Hampton, 2002), welche ihre Gegenstück in dem putativen E3-Komplex aus Pex2p, Pex10p und Pex12p finden. An beiden Systemen ist das 26S Proteasom für Polyubiquitin-vermittelte Degradation beteiligt.

Ein weitläufigerer Zusammenhang besteht mit den sog. *i*-AAA und *m*-AAA Proteinen der inneren Mitochondrienmembran (Langer *et al.*, 2001), VAR2 der Chloroplasten (Chen *et al.*, 2000) sowie mit FtsH der Bakterienmembran (Ito & Akiyama, 2005). Auch diese AAA-ATPasen sind in der Lage Proteine aus Membranen zu extrahieren. Jedoch besitzen sie selber eine zusätzliche Proteasefunktion, weshalb diese Extraktions- und Degradationssysteme prokayontischen Ursprungs entsprechend ohne Ubiquitin oder separate Proteasen, wie das

26S Proteasom, arbeiten. Allen genannten AAA-ATPasen gemein ist jedoch die Fähigkeit Proteine aus Membranen zu extrahieren.

Die Ähnlichkeit in der Proteinzusammensetzung der ERAD-Maschinerie und der peroxiosmalen Matrixproteinimportkomplexe scheint, wie aktuelle phylogenetische Studien auf molekularer Ebene belegen, einen gemeinsamen evolutionären Ursprung zu haben (Gabaldon *et al.*, 2006; Schlüter *et al.*, 2006). So sind Pex1p, Pex6p und Cdc48p tatsächlich auf eine charakteristische Gruppe von ursprünglichen AAA-ATPasen zurückzuführen. Auch die RING-Finger Peroxine Pex2p, Pex10p und Pex12p leiten sich hiernach zusammen mit Hrd1p von frühen RING-Proteinen ab. Ähnliches gilt für die E2-Enzyme Pex4p und Ubc7p.

Auch wenn sich die Aufgaben der Systeme unterscheiden – Retrotranslokation zum Zytosol und Degradation auf der einen Seite und Matrixproteinimport gekoppelt mit Rezeptor-Recycling auf der anderen Seite – lassen sich beide Systeme aufgrund ihrer prinzipiellen Module und Mechanismen als Ubiquitin vermittelte Zielsteuerungssysteme verstehen.

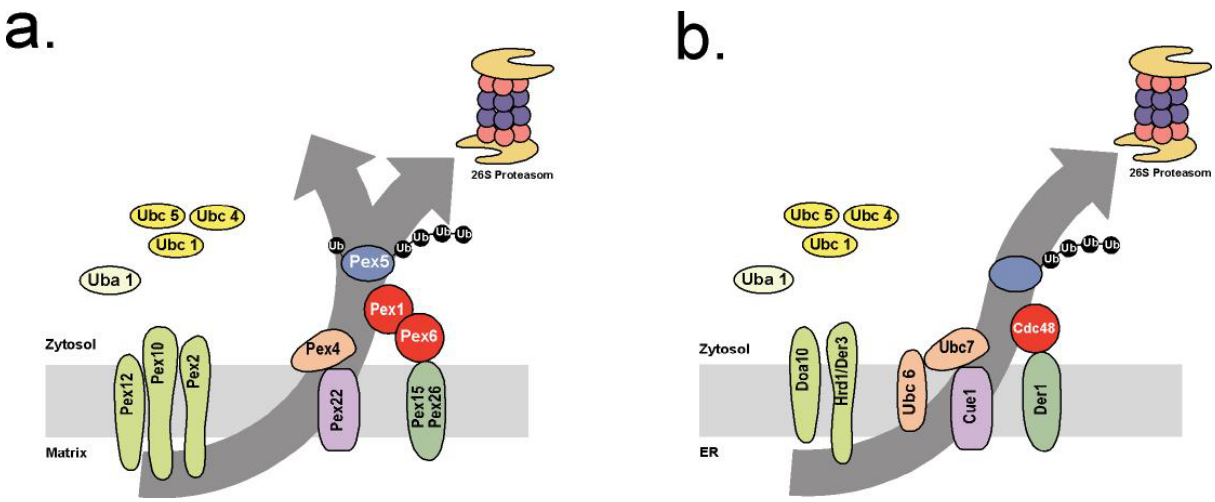

Abb. 3.4 Die Transportsysteme für (a) den Export von Pex5p aus dem Peroxisom und (b) für die Retrotranslokation von Proteinen aus dem ER weisen Ähnlichkeiten auf. Beide Systeme Teilen sich Komponenten des Ubiquitin-Systems, wie das E1 (Uba1p) und die zytosolischen E2-Enzyme Ubc1p, Ubc4p und Ubc5p. Beide Systeme beinhalten je eines der einzigen beiden E2-Enzyme, die einen Membrananker besitzen (Peroxisom: Pex4p an Pex22p / ER: Ubc7p an Cue1p; zusätzlich Ubc6p). Die peroxisomlen RING-Finger Proteine Pex2p, Pex10p und Pex12p sind putative E3 Enzyme, während die RING Finger Doa10p und Hrd1p des ER nachgewiesene E3-Ligasen sind. Die Membranproteine Pex15p (*Sc*) bzw. Pex26p (*Hs*) verankern den heterohexameren AAA-Komplex bestehend aus Pex1p und Pex6p an der peroxisomale Membran. Das Homohexamere AAA-Protein Cdc48p bindet über Der1p an die ER Membran. In beiden Systemen wird polyubiquitinyliertes Substrat von dem AAA-Komplex aus der Membran extrahiert und dem 26S Proteasom zur Degradation übermittelt. Monoubiquitinyliertes Substrat findet sich nur bei den Peroxisiomen und dient vermutlich dem Recycling. (aus Platta *et al.*, 2006b, verändert).

Die in der vorliegenden Arbeit diskutierten Experimente reihen sich in eine Folge von *in vitro* Untersuchungen zum peroxisiomalen Matrixproteinimport ein und vermögen dazu beizutragen eine wichtige mechanistische Lücke zu schließen.

In ersten Studien konnte demonstriert werden, dass der Import von radioaktiv markierten peroxisomalen Matrixproteinen in Peroxisomen aus Rattenleber ein von Temperatur und ATP-Hydrolyse abhängiger Prozeß ist (Fujiki & Lazarow, 1985; Imanaka *et al.*, 1987; Wendland & Subramani, 1993). Dieser ATP-abhängige Schritt wurde weiter charakterisiert so dass festgestellt werden konnte, dass sich die subzelluläre Verteilung von Pex5p ebenfalls in Abhängigkeit von Temperatur und ATP verändert (Dodt & Gould, 1996). Diese Studien in permeabilisierten menschlichen Fibroblasten lieferten die ersten Beweise, dass Pex5p unter den gleichen Bedingungen, in denen der Matrixproteinimport gestört ist, reversibel in Peroxisomen akkumuliert (Dodt & Gould, 1996). Detaillierte Untersuchungen in einem zellfreien System mit *in vitro* translatiertem, radioaktiv markiertem Pex5p und Rattenleber Peroxisomen demonstrierten, dass die Anbindung und Translokation von Pex5p ATP-unabhängig stattfindet, jedoch der Export von Pex5p den ATP-Hydrolyse abhängigen Schritt darstellt. Dabei verhält sich die mit Pex14p komplexierte Pex5p-Population wie ein integrales Membranprotein (Gouveia *et al*., 2000; Gouveia *et al.*, 2003; Oliveira *et al.*, 2003).

Zusammengefaßt ergeben diese Daten das Bild, dass der ATP-abhängige Export von Pex5p den limitierenden Schritt des peroxisomalen Matrixproteinimports darstellt. Die Bindungskapazitäten für Pex5p scheinen unter Wildtyp Bedingungen so gut wie gesättigt zu sein, so dass, als vereinfachte Idee, für jeden Kargo-beladenen PTS1-Rezeptor ein Kargo-entladenes Pex5p die Membran verlassen muß, um einen optimalen Fluß des Proteinimports zu gewährleisten.

Die Identität dieser ATPase blieb lange Zeit unklar, bis im Rahmen dieser Arbeit der Zusammenhang zu Pex1p und Pex6p hergestellt werden konnte (Platta *et al.*, 2005). Die entscheidende Neuerung des *in vitro* Ansatzes bestand darin, dass mit Hilfe der Hefe-Genetik eine Vielzahl an Mutanten generiert und getestet werden konnte, was das System sehr variabel gestaltet. Auf diese Weise war es möglich, die 1985 beobachtet Abhängigkeit des peroxisomalen Matrixproteinimports von ATP-Hydrolyse auf die Aminosäuren D797 in dem Hydrolyse-Motiv von Pex1p bzw. D831 in Pex6p zu reduzieren (Platta *et al.*, 2005).

Die Abhängigkeit des Pex5p-Exportes von Pex1p, Pex6p und Pex26p konnte später beim Menschen anhand eines auf Patientenzellen beruhenden zellfreien *in vitro* Systems bestätigt werden (Miyata & Fujiki, 2005).

Der exakte Mechanismus der Erkennung von Pex5p durch die AAA Peroxine ist weitgehend unklar. Die ersten 17 Aminosäuren des zum Zytosol weisendem N-Terminus von integralem menschlichen Pex5p sind *in vitro* essentiell für dessen Ablösung (Costa-Rodriguez *et al.*, 2004). Ein N-terminal um 19 Aminosäuren verkürztes Pex20p Konstrukt akkumuliert in der peroxisomalen Membran von *P. pastoris* (Leon *et al.*, 2006a), was auf einen vergleichbaren Mechanismus für alle PTS-Rezeptoren schließen läßt. Innerhalb dieser ersten Aminosäuren befindet sich ein in allen PTS-Rezeptoren konserviertes Motiv (Cys-X_n-Asn-(Ala/Gly)-(Leu/Ala)), dessen Funktion unverstanden ist. Es wäre somit denkbar, dass sich hier die hypothetische Bindestelle der AAA-Peroxine an Pex5p befindet. Obwohl Pex5p Teil des AAA-Komplexes ist (Platta *et al.*, 2005-Fig.1b), konnte bisher keine direkte Bindung von Pex1p oder Pex6p mit dem PTS1-Rezeptor zweifelsfrei bewiesen werden, so dass eventuell ein zusätzlicher Faktor diese Bindung vermitteln könnte. Unter Berücksichtigung der unter 3.4 diskutierten Monoubiquitinylierung von Pex5p durch Pex4p, wäre es denkbar, dass diese Bindung über Ubiquitin vermittelt wird.

In diesem Zusammenhang war es von Bedeutung, die Funktion der Ubiquitinylierung von Pex5p während der Export-Reaktion zu untersuchen.

Die *in vitro* Dislokations-Experimente veranschaulichten, dass die Polyubiquitinylierung des PTS1-Rezeptors nicht notwendig für dessen Rezeptorzyklus ist, da sowohl das *myc*Pex5p als auch das Pex5p(K18R;K24R) wie das Wildtyp Pex5p zyklisieren (Platta *et al.*, 2006c-Fig.1b + 3). Zur Untersuchung der Bedeutung von Monoubiquitinylierung wurde ein Pex4p-freies *in vitro* System benutzt, in welchem Pex5p nicht mehr monoubiquitinyliert werden kann (Platta *et al.*, 2006c-Fig.3). Die Verhinderung der Monoubiquitinylierung reduzierte die Menge an freigesetztem Wildtyp Pex5p drastisch. Dieser Rest-Export war abhängig von Pex1p. Die Kombination aus dem Fehlen der Monoubiquitinylierung (*pex4*Δ) mit dem Fehlen der Polyubiquitinylierung (*myc*Pex5p, Pex5p(K18R;K24R)) resultierte in einer vollständigen Blockierung der Exportreaktion. Der zu beobachtende restliche Export von Pex5p in dem Pex4p-defizienten System scheint somit über die Polyubiquitinylierung vermittelt zu sein.

Eine Darstellung der Monoubiquitinylierung während der Export Reaktion war aus methodischen Gründen nicht möglich, da das NEM neben den deubiquitinylierenden Enzymen auch die AAA-Peroxine inhibiert (Platta, unpubliziert). Eine alternative

Darstellungsmethode wird erarbeitet, indem Ubiquitin-Aldehyd anstelle von NEM Verwendung finden soll, da es konjugiert, aber nicht deubiquitinyliert werden kann und zudem keinen direkten Einfluss auf die AAA-Peroxine ausübt.
Zusammengefaßt läßt sich ein hierarchisch gestuftes Bild der Ubiquitin-Abhängigkeit für die Dislokation von Pex5p feststellen. Das zentrale Signal scheint hierbei die Monoubiquitinylierung von Pex5p darzustellen. Wenn keine Monoubiquitinylierung stattfinden kann, wird ein kleinerer Teil von polyubiquitinyliertem Pex5p exportiert. Fehlen beide Modifikationstypen, findet keine *in vitro* Export Reaktion mehr statt.

Die Weise, wie die Ubiquitinylierung die Dislokation von Pex5p fördert, bleibt noch zu untersuchen. Die Ubiquitinylierung wird vermutlich benötigt, um die Kargo-entladenen Rezeptoren zu markieren und diese von den noch Kargo-beladenen Rezeptoren zu unterscheiden. Die AAA-Peroxine mögen direkt über eine putative Ubiquitin-Bindedomäne mit dem Ubiquitin interagieren, oder aber indirekt über noch zu identifizierende spezifische Adaptoren, welche für viele AAA-Proteine beschrieben worden sind (zur Übersicht Erzberger & Berger, 2006).
Es wäre auch denkbar, dass die Ubiquitinylierung eine Konformationsänderung im N-Terminus von Pex5p hervorruft, die dann erst die Bindetaschen für die AAA-Peroxine zugänglich macht. Der N-terminale Bereich ist eine weitgehend ungefaltete Domäne, wodurch dieser sich in der Lage befindet, sehr dynamisch reagieren und interagieren zu können. (Carvalho *et al.*, 2006). Dieses Modell könnte gewissermaßen das Gegenstück zum „Docking"-Mechanismus von Pex5p sein, für welchen Hinweise existieren, dass die Pex14p-Bindebereiche auf Pex5p erst nach Kargo-Bindung zugänglich sind (Gouveia *et al.*, 2003).
Nicht zuletzt ist auch ein dualer Bindemechanismus, wie er für Cdc48p (p97) beschrieben worden ist (Ye *et al.*, 2003) möglich, nach dem zuerst als Erkennungssignal das Ubiquitin und anschließend ein nicht-modifiziertes Segment des Zielproteins gebunden wird, um den Halt für die eigentliche Retrotranslokation zu gewährleisten. Dieses Modell würde die Bedeutung der ersten 17 Aminosäuren im menschlichen Pex5p miteinbeziehen.

Festzuhalten ist jedoch, dass die einzelnen in den vergangenen Jahren dokumentierten Beobachtungen bezüglich der „späten Schritte" des peroxisomalen Matrixproteinimports durch die in dieser Arbeit dokumentierte Beschreibung und funktionelle *in vitro* Analyse der Komponenten (AAA-Komplex, Pex4p/Pex22p-Komplex) wie auch der Signale (Mono- und Polyubiquitinylierung) der Rezeptor-Export-Maschinerie in ein kohärentes mechanistisches

Bild eingefügt werden können (Abb. 3.5). Sie liefert auch die molekulare Funktion für PEX1, dem Gen, das in fast 80 % aller Zellweger-Patienten mutiert vorliegt. Neben der Identifizierung der AAA-Peroxine als essentielle Motorprotein für die Freisetzung von Pex5p konnte die funktionelle Bedeutung der Ubiquitinylierung des Rezeptors demonstriert werden, wobei die wichtigste Form die Pex4p-vermittelte Monoubiquitinylierung darstellt. Somit kann die Energie-Abhängigkeit des Exportschrittes des PTS1-Rezeptors in zwei distinkte Reaktionen aufgeteilt werden: E1/E2/E3-abhängige Ubiquitinylierung und AAA-abhängige Dislokation (Erdmann & Schliebs, 2005; Platta *et al*., 2006b).

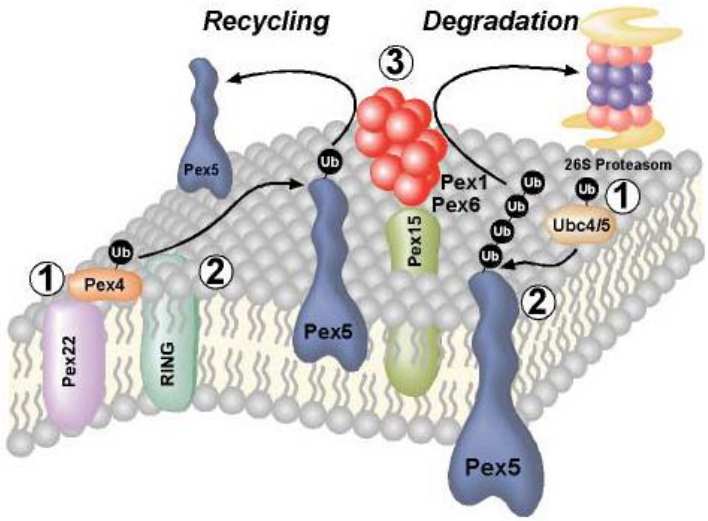

Abb.3.5 Modell zum molekularen Mechanismus des Pex5p Exports. Das integrale Pex5p kann entweder durch Pex4p monoubiquitinyliert werden, oder durch Ubc4p bzw. Ubc5p Polyubiquitinketten erhalten. An beiden Prozessen mögen die RING-Finger Peroxine als putative E3-Ligasen beteiligt sein. Der über Pex15p verankerte Pex1p/Pex6p-Komplex löst in beiden Fällen das modifizierte Pex5p aus der Membran. Polyubiquitinyliertes Pex5p wird proteasomaler Degradation zugeführt, während monoubiquitinyliertes Pex5p deubiquitinyliert und recycled wird. (aus Platta *et al*., 2006b, verändert).

Die Ermittlung des genauen molekularen Mechanismus, über welchen das Ubiquitin den Export von Pex5p vermittelt, wird der nächste Schritt zum Verständnis des peroxisomalen Proteintransportsystems sein. Letztendlich soll verstanden werden, wie Ubiquitinylierung und Dislokation der Rezeptoren mechanistisch mit der Ablösung der Kargo-Proteine verbunden ist.

4. LITERATURVERZEICHNIS

(für Kapitel 1 und Kapitel 3)

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Veröffentlichungen in wissenschaftlichen Journalen:

Platta, H.W., Girzalsky, W. & Erdmann, R. (2004)
Ubiquitination of the peroxisomal import receptor Pex5p.
Biochemical Journal **384**, 37-45.

Platta, H.W., Grunau, S., Rosenkranz, K., Girzalsky, W. & Erdmann, R. (2005)
Functional role of the AAA peroxins in dislocation of the cycling PTS1-receptor back to the cytosol.
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Meeting Report: Life of proteins.
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The Journal of Biological Chemistry **281**, 27003-27015.

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Ubiquitination of the peroxisomal import receptor Pex5p is required for its recycling. *(eingereicht)*

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Ausgewählte Vorträge:

Platta, H.W., Rosenkranz, K., Girzalsky, W. & Erdmann, R.
Pex5p-Ubiquitination: Pex1p/Pex6p-mediated dislocation of membrane associated Ub-Pex5p.
International Meeting on the Topogenesis of Organellar Proteins
Bochum, 28.10.-30.10.2004

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44th Meeting of the American Society of Cell Biologists
Washington, DC, (USA) 4.12.-8.12.2004
(Molecular Biology of the Cell **15** Suppl., 12a)

Platta, H.W., Grunau, S., Rosenkranz, K., Girzalsky, W. & Erdmann, R.
Peroxisomal protein import: functional role of the AAA-peroxins in dislocation of the cycling PTS1-receptor back to the cytosol.
6th International Conference on AAA-Proteins
Graz (Österreich), 14.09.-17.09.2005

Platta, H.W., Grunau, S., Girzalsky, W. & Erdmann, R.
AAA-peroxins Pex1p and Pex6p are dislocases, mediating the release of the cycling PTS1-receptor from the peroxisomal membrane to the cytosol.
Peroxisome Meeting
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Platta, H.W.:
Functional characterization of the Pex5p receptor cycle.
Open European Peroxisome Meeting
Leuven (Belgien), 18.09.-19.09.2006

Ausgewählte Posterpräsentationen:

Harald W. Platta, Silke Grunau, Wolfgang Girzalsky & Ralf Erdmann:
Ubiquitination and Pex1p/Pex6p-dependend dislocation of Pex5p.
International Meeting on the Topogenesis of Organellar Proteins
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Harald W. Platta, Silke Grunau, Stefan Franke, Wolfgang Girzalsky & Ralf Erdmann:
Ubiquitination and AAA-peroxin dependent dislocation of Pex5p back to the cytosol.
28th Annual Meeting of the German Society for Cell Biology (DGZ)
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Harald W. Platta, Silke Grunau, Wolfgang Girzalsky & Ralf Erdmann:
AAA-peroxins Pex1p and Pex6p are dislocases, mediating the release of the cycling PTS1-receptor from the peroxisomal membrane to the cytosol.
6th International Conference on AAA-Proteins
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Function of the AAA-peroxins Pex1p and Pex6p in the cycle of the receptor Pex5p.
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Function of the AAA-peroxins Pex1p and Pex6p in the cycle of the receptor Pex5p.
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Harald W. Platta, Silke Grunau, Wolfgang Girzalsky & Ralf Erdmann:
AAA-peroxins Pex1p and Pex6p are dislocases, mediating the release of the cycling PTS1-receptor from the peroxisomal membrane to the cytosol.
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Wolfgang Girzalsky, Silke Grunau, Harald W. Platta, Katja Rosenkranz, & Ralf Erdmann:
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Harald W. Platta, Daniel Schlee, Fouzi El Magraoui, Astrid Korneli, Silke Grunau, Wolfgang Girzalsky & Ralf Erdmann:
Functional characterization of the Pex5p receptor cycle.
Open European Peroxisome Meeting
Leuven, (Belgien) 18.09.-19.09.2006

6. ANHANG

ABKÜRZUNGSVERZEICHNIS

AAA	*ATPases associated with various cellular Activities*
ABC	*ATP-binding cassette*
ATP	Adenosintriphosphat
cim	*co-lethal in mitogenesis*
DUB	deubiquitinylierendes Enzym
ERAD	*endoplasmatic reticulum associated degradation*
GFP	Grün-fluoreszierendes Protein
E1	Ubiquitin aktivierendes Enzym
E2	Ubiquitin-konjugierendes Enzym
E3	Ubiquitin-Protein-Isopeptid Ligase
E. coli	*Escherichia coli*
ER	Endoplasmatisches Retikulum
fox	*fatty acid oxidation*
IRD	Infantile Refsum Krankheit
min	Minuten
mPTS	peroxisomales Membran-Sortierungssignal
NALD	Neonatale Adrenoleukodystrophie
NSF	*N-ethylmaleimide sensitive fusion protein*
Onu	*oleic-acid-non-utilizer*
PBD	p*eroxisome biogenesis disorder*
pas	*peroxisome assembly*
pex	*peroxisome assembly*
RCDP	Rhizomelische Chondrodysplasia punctata
PMP	Peroxisomale Membranproteine
PNS	*post nuclear supernatant*
ProtA	ProteinA
PTS	Peroxisomales „*Targeting*“ (Sortierungs)-Signal
RING	*really interesting new gene*
SH3	*Src homology3*
SNAP	*soluble NSF attachement protein*
SNARE	SNAP-Rezeptoren
SUMO	*small ubiquitin-like modifier*
TCA	Trichloressigsäure
TEV	*tobacco etch virus*
Ub	Ubiquitin

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und bei keiner anderen Fakultät eingereicht und dass ich keine anderen als die angegebenen Hilfsmittel verwendet habe. Es handelt sich bei der heute von mir eingereichten Dissertation um fünf in Wort und Bild völlig übereinstimmende Exemplare.
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Bochum, den 16. Oktober 2006