Cell. Mol. Life Sci. 63 (2006) 1686–1699
1420-682X/06/141686-14
DOI 10.1007/s00018-006-6110-y

Cellular and Molecular Life Sciences

Research Article

Farnesylation of Pex19p is not essential for peroxisome biogenesis in yeast and mammalian cells

I. M. K. Vastiau [a], E. A. Anthonio [a], M. Brams [a], C. Brees [a], S. G. Young [b], S. Van de Velde [c, d], R. J. A. Wanders [e], G. P. Mannaerts [a], M. Baes [f], P. P. Van Veldhoven [a] and M. Fransen [a, *]

[a] Laboratorium voor Farmacologie, Departement Moleculaire Celbiologie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, Campus Gasthuisberg (O/N 1), Herestraat 49 bus 601, 3000 Leuven (Belgium), Fax: +32 16 345699, e-mail: marc.fransen@med.kuleuven.be
[b] Department of Medicine, Division of Cardiology, University of California, Los Angeles, California (USA)
[c] Laboratorium voor Moleculaire Celbiologie, Departement Biologie, Faculteit Wetenschappen, Katholieke Universiteit Leuven, Leuven (Belgium)
[d] Departement Moleculaire Microbiologie, Vlaams Interuniversitair Instituut voor Biotechnologie, Leuven (Belgium)
[e] Laboratorium Genetisch Metabole Ziekten, Departement voor Pediatrie, Academisch Medisch Centrum, Universiteit van Amsterdam, Amsterdam (The Netherlands)
[f] Laboratorium voor Klinische Chemie, Faculteit Farmaceutische Wetenschappen, Katholieke Universiteit Leuven, Leuven (Belgium)

Received 10 March 2006; received after revision 28 April 2006; accepted 30 May 2006
Online First 22 June 2006

Abstract. Pex19p exhibits a broad binding specificity for peroxisomal membrane proteins (PMPs), and is essential for the formation of functional peroxisomal membranes. Pex19p orthologues contain a C-terminal *CAAX* motif common to prenylated proteins. In addition, *Saccharomyces cerevisiae* and Chinese hamster Pex19p are at least partially farnesylated *in vivo*. Whether farnesylation of Pex19p plays an essential or merely ancillary role in peroxisome biogenesis is currently not clear. Here, we show that (i) nonfarnesylated and farnesylated human Pex19p display a similar affinity towards a select set of PMPs, (ii) a variant of Pex19p lacking a functional farnesylation motif is able to restore peroxisome biogenesis in Pex19p-deficient cells, and (iii) peroxisome protein import is not affected in yeast and mammalian cells defective in one of the enzymes involved in the farnesylation pathway. Summarized, these observations indicate that the *CAAX* box-mediated processing steps of Pex19p are dispensable for peroxisome biogenesis in yeast and mammalian cells.

Keywords. Peroxisomes, biogenesis, peroxins, protein import, Pex19p, farnesylation.

Introduction

Peroxisomes play a pivotal role in normal human development [1]. During the last 15 years, tremendous progress has been made in the identification of proteins involved in peroxisome biogenesis [2]. Comprehensive interaction analyses have shown that these proteins, collectively called peroxins (abbreviated Pexp and followed by a number corresponding to the order of discovery [3]), form a highly interconnected protein network [4–7]. This network can be further defined into distinct functional modules [8, 9]. For example, in *Pichia pas-*

* Corresponding author.

toris and *Saccharomyces cerevisiae*, the peroxisome targeting signal (PTS)-receptor docking subcomplex, consisting of the peroxins Pex14p, Pex17p, and a portion of Pex13p, and the putative translocation subcomplex containing Pex2p, Pex10p, and Pex12p, form a large complex that is essential for matrix protein import [10, 11]. However, at present only little is known about how these and other peroxin-peroxin interactions and complexes are regulated.

Protein-protein interactions can be controlled by either altering their effective local concentrations or by influencing their binding affinities. Covalent modifications may act as powerful molecular switches that trigger the assembly or disassembly of protein complexes [12]. At present, six peroxins have been reported to undergo post-translational modifications: Pex5p, the import receptor for peroxisomal matrix proteins containing a C-terminal PTS, and Pex18p and Pex20p, which are two components necessary for Pex7p-mediated peroxisomal import of matrix proteins containing an N-terminal PTS, can be mono-, di- and/or polyubiquitinated in *S. cerevisiae* [13–16] and *P. pastoris* [17]; Pex14p, the PTS-receptor docking protein, has been shown to be at least partially phosphorylated in *Hansenula polymorpha* [18], *P. pastoris* [19] and mammals (I. Vastiau and M. Fransen, unpublished results); a portion of Pex15p, a PMP, is phosphorylated in *S. cerevisiae* [20]; and Pex19p, a protein essential for the biogenesis of numerous PMPs, which can be farnesylated in *S. cerevisiae* [21] and mammals [22, 23]. To understand the biological function of these covalent peroxin modifications remains a significant challenge. Recently, it has been postulated that monoubiquitination of Pex5p may be required for the recycling of Pex5p from the peroxisome, while polyubiquitination may function as a disposal mechanism for Pex5p when it gets trapped or immobilized in the import pathway [15, 16]. Also Pex18p and Pex20p ubiquitination has been speculated to play a role in receptor recycling and/or turnover [13, 17]. Regarding the biological role of phosphorylation of Pex14p and Pex15p, only little is known [18–20], and conflicting data exist on the role of farnesylation of Pex19p in peroxisome biogenesis (see below) [21, 23–25].

With the exception of the trypanosomatid orthologues, all currently known Pex19p proteins contain a farnesylation consensus motif at their C-terminus [26, 27]. This motif, also called the *CAAX* box (where C is cysteine, A is an aliphatic amino acid, and X is glutamine, cysteine, serine, alanine, or methionine), directs a series of three post-translational modifications: the covalent attachment of a farnesyl group to the cysteine in the *CAAX* motif; proteolysis of the three C-terminal (*AAX*) amino acids; and methylation of the newly exposed farnesylcysteine residue [28]. Addition of the 15-carbon prenoid group and carboxyl methylation significantly augments the hydrophobicity of the *CAAX*-containing proteins [29]. This increase in hydrophobicity may alter the protein's subcellular localization and/or binding properties [30]. In this context, it should be pointed out that farnesylated Pex19p has been reported to be anchored in the peroxisome membrane [23]. Also, it has been shown that the *CAAX* box of yeast and human Pex19p is an important determinant in the affinity of Pex19p for ScPex3p [21], HsPex10p, HsPex12p, and HsPex13p [31]. These findings are in line with the observation that the *CAAX* box of Pex19p is essential for the proper function of the protein [21, 23]. However, this conclusion is in direct conflict with other studies suggesting farnesylation has an ancillary rather than a central role in Pex19p function. That is, it has been reported that (i) the peroxisome docking domain of human Pex19p resides in the N-terminal, and not the C-terminal domain of Pex19p [32, 33], (ii) both farnesylated and nonfarnesylated Pex19p display a high binding affinity for the adrenoleukodystrophy protein (ALDP) *in vitro* [34], and (iii) the *CAAX* box of Pex19p is – at least under conditions of overexpression – dispensable for Pex19p function [5, 25]. Here we provide substantial new information that none of the *CAAX*-directed post-translational modifications of Pex19p are essential for peroxisomal protein import in yeast and mammalian cells.

Materials and methods

Plasmids and strains. The oligonucleotides (Invitrogen) constructed for this study are compiled in Table 1. Cloning vectors were obtained from Clontech (pEGFP-C1, pEGFP-N1, pGAD424), Promega (PinPoint Xa1), Qiagen (pQE30), Dr. A. Hartig (University of Vienna, Austria) (pJR233), Dr. G. Stier (EMBL, Heidelberg, Germany) (pETM11), and Dr. J. Thevelein (K. U. Leuven, Leuven, Belgium) (YCplac33). PCR applications were performed routinely using *Pfx* DNA polymerase (Invitrogen). Restriction enzymes were purchased from TaKaRa or Invitrogen. The *Escherichia coli* strain Top10F' (Invitrogen) was used for all DNA manipulations. To generate pMB1, a mammalian expression plasmid coding for the peroxisomal marker protein EGFP-PTS1, two 25-mers (KSKL.Fw and KSKL.Rv) were allowed to hybridize and were ligated into *BsrG* I/*Not* I-restricted pEGFP-N1. To construct pMF790, a mammalian expression plasmid coding for the plasma membrane marker protein EGFP-C-HA-Ras$_{(170–189)}$, the oligonucleotides PalmFarn.Fw and PalmFarn.RV were allowed to hybridize and ligated into *Bgl* II/*Sal* I-restricted pEGFP-C1. Mammalian expression vectors coding for EGFP-HsPex19p$_{\Delta CAAX}$ (pMF125), EGFP-HsPex19p$_{C296S}$ (pIV23), and wild-type HsPex19p (pMF1245) were also constructed. Therefore, the corresponding cDNAs were (i) amplified by PCR (template: pMF134 [31]; primer pairs: Pex19.4 and Pex19.5 (pMF125), Pex19$_{C296S}$.Rv and Pex19.5 (pIV23), HsPex19.

Table 1. Synthetic oligonucleotide primers used in this study (restriction sites are underlined).

Name	Nucleotide sequence
19.BTH2	5′-gaggggtacctcacatgatcagacactg-3′
FTbF2	5′-ggaatgtgaagatgaggtgac-3′
FTbR2	5′-gagaggatgtgactcggttttc-3′
Fw(-362)SphI	5′-acatacatgcatgccaaatggatgatcctttctcc-3′
$Gal4_{AD1}$	5′-ggggatccatgaagctactgtcttctatc-3′
$Gal4_{AD2}$	5′-ccctgcagctgtctttgacctttgttac-3′
HsPex19.fwSalI	5′-gcggagtcgaccaagatggccgccgctgag-3′
KSKL.Fw	5′-gtacaagagcaagctgtaagcgcgc-3′
KSKL.Rv	5′-ggccgcgcgcttacagcttgctctt-3′
PalmFarn.Fw	5′-gatctctgaaccctcctgatgagagtggccccggctgcatgagctgcaagtgtgtgctctcctgactgca-3′
PalmFarn.Rv	5′-gtcaggagagcacacacttgcagctcatgcagccggggccactctcatcaggagggttcaga-3′
Pex13.8	5′-atctgcagagattttgctgaggtagctgc-3′
Pex13.155Fw	5′-agagagccatggcgtataacagtttcagggctg-3′
Pex19.4	5′-tgtctcgagctgttcaccactggcacctgg-3′
Pex19.5	5′-atgggatccatggccgccgctgaggaagg-3′
$Pex19_{C296S}$.Rv	5′-gtaagctttcacatgatcagagactgttc-3′
RNAi oligo 532	5′-gcacagaggaagccuacaacgucau-3′
RNAi oligo 1006	5′-cccucagcaugagccauuggauguu-3′
Rv(+368)EcoRI	5′-ccggaattccattttcgcgtggtcgagag-3′
$ScPex19_{C347S}$.FwXhoI	5′-ggtagcaaacaacaataactcgagtataattatgataccagtaaa-3′
$ScPex19_{C347S}$.RvXhoI	5′-ctcgagttattgttgtttgctaccgtcggttaattcc-3′

fwSalI and 19.BTH2 (pMF1245)), (ii) gel purified, (iii) digested with *Bam* HI/*Sal* I (pMF125), *Bam* HI/*Hind* III (pIV23) or *Sal* I/*Kpn* I (pMF1245), and (iv) subcloned into the *Bgl* II/*Sal* I-digested pEGFP-C1 (pMF125), *Bgl* II/*Hind* III-digested pEGFP-C1 (pIV23), or *Xho* I/*Kpn* I-digested pEGFP-N1 vector (pMF1245). An amplicon encompassing the full-length *ScPEX19* open reading frame as well as its natural promoter was generated by PCR, using yeast genomic DNA in combination with the primers Fw(-362)SphI and Rv(+368)EcoRI. The purified PCR product was digested with *Sph* I and *Eco* RI, and subcloned into the *Sph* I/*Eco* RI-restricted yeast expression vector YCplac33 (pIV19). A yeast expression construct coding for $ScPex19p_{C347S}$ (pIV20) was generated by fusion PCR. In a first PCR reaction, two PCR fragments (template: pIV19; primers: $ScPex19_{C347S}$.FwXhoI and Rv(+368)EcoRI (fragment 1), $ScPex19_{C347S}$.RvXhoI and Fw(-362)SphI (fragment 2)) were generated. These PCR fragments were fused and used as template in a second PCR reaction [primers: Fw(-362)SphI and Rv(+368)EcoRI]. After digestion with *Sph* I and *Eco* RI, the fusion fragment was subcloned into the *Sph* I/*Eco* RI-digested YCplac33 vector. The yeast expression vector coding for ScPex11p-EGFP (pMF959) was constructed by transferring the *Hind* III/*Eco* RI fragment of pEW174 (gift of Dr. B. Distel, University of Amsterdam, The Netherlands) into pJR233 digested with the same restriction enzymes. To generate bacterial expression constructs coding for $(His)_6$-$HsPex13p_{(155–233)}$ (pMF910) and $(His)_6$-$Gal4p_{AD}$ (pMF349), (i) the corresponding cDNAs were amplified by PCR (pMF910: template pMF103; primers Pex13.155Fw and Pex13.8; pMF349: template pGAD424, primers $Gal4_{AD1}$ and $GAL4_{AD2}$), (ii) digested with *Nco* I and *Pst* I (pMF910) or *Bam* HI and *Pst* I (pMF349), and (iii) cloned into the *Nco* I/*Pst* I-digested pETM11 (pMF910) or *Bam* HI/*Pst* I-digested pQE30 vector (pMF349). To generate the bacterial expression construct coding for biotinylated $HsPex13p_{(155–233)}$ (pMF357), the *Bgl* II/*Sma* I-digested fragment of pMF593 [31] was cloned into the *Bam* HI/*Sma* I-digested PinPoint Xa1 vector. The identities of the constructs listed above were confirmed by DNA sequencing (Agowa). The plasmids encoding $(His)_6$-$HsPex5pRpA_{(1–624)}$ (pLA145) [35], $Gal4p_{BD}$-HsPex13p (pMF103) [31], EGFP-HsPex13p (pMF121) [31], $(His)_6$-HsPex14p (pKG52) [36], HsPex19p (pMF134) [31], GST-HsPex19p (pTW299) [31], DsRed-PTS1 (pMF578) [31], EGFP-PTS1 (pJR233) [37], biotinylated RnDCR-AKL (pMF45) [38], and biotinylated HsPex14p (pMF42) [39] are described elsewhere. The haploid *S. cerevisiae* strain BY4741 (genotype: MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; accession number: Y00000), the corresponding null mutants of *pex19*, *ram1*, *rce1*, *ste24*, and *ste14* (open reading frame::kanMX4) (accession numbers Y03762, Y03787, Y00860, Y06920, and Y04246, respectively), and the diploid *S. cerevisiae* BY4743 strains homozygous (accession number: Y33787) or heterozygous (accession number: Y23787) for null alleles of *RAM1*, were obtained from Euroscarf. The yeast two-hybrid host strain

SFY526 was obtained from Clontech. Bacterial and yeast cells were transformed and selected as described [6] (see also 'Matchmaker Library Protocol'; Clontech). Yeast cells deficient in farnesyltransferase (FTase) were always cultivated at room temperature. To assay yeast strains for growth in the presence of oleate as the sole carbon source, the cells were pelleted, washed, and resuspended in sterile water to an optical density of 0.4 at 600 nm. Five microliter aliquots (≈ 10 000 colony-forming units) were plated on oleic acid plates containing 0.1% (v/v) oleic acid, 0.4% (v/v) Tween-40, 0.1% (w/v) yeast extract (Difco), 2% (w/v) agar, and synthetic dropout medium [20] (see also 'Matchmaker Library Protocol').

Cell culture, transfections, drugs, and (immuno)fluorescence microscopy. Chinese hamster ovary (CHO) cells, human Pex19p-deficient fibroblasts [23], and immortalized mouse fibroblasts ($Rce1^{+/+}$, $Rce1^{-/-}$, $Icmt^{+/+}$, $Icmt^{-/-}$) [40, 41] were cultured as described elsewhere [31]. Cells seeded in a 6-well plate (RNA isolation) or onto glass coverslips in a 12-well plate (immunocytochemical staining) were grown to 30–50% (Stealth™ RNAi oligonucleotides) or 60–70% (plasmid DNA) confluency, and transiently transfected using polyethylenimine (plasmid DNA, CHO cells) [42], Lipofectamine Plus (plasmid DNA, fibroblasts) (Invitrogen), or Lipofectamine 2000 (Stealth™ RNAi oligonucleotides, fibroblasts) (Invitrogen). The FTase inhibitor FTI-277 (Calbiochem) and the geranylgeranyltransferase I inhibitor GGTI-298 (Calbiochem) were initially dissolved in DMSO, diluted with αMEM complete medium (BioWhittaker) supplemented with 400 µM dithiothreitol (DTT), and added to the cells 3 h post-transfection. At 3 days after transfection, the cells were processed for (in)direct fluorescence microscopy as described elsewhere [38]. The peroxisomal localization of the EGFP-fusion proteins was confirmed by colocalization studies with Pex14p [39], PMP70 (Zymed Laboratories), or the peroxisome-targeted DsRed-PTS1 reporter protein [31]. Fluorescence was observed under a Leica DMR microscope equipped with FITC/RSGFP/Bodipy/Fluo3/DIO and Texas Red filters. Colocalization of two signals was performed with Lucia G on DXM1200 version 4.71 software (Analis SA).

RNA interference. The BLOCK-iT™ RNAi Designer software (Invitrogen) was used to design two Stealth™ RNAi (Invitrogen) molecules (Table 1) potentially capable of down-regulating the expression of the β-subunit of mouse FTase. *Rce1*$^{+/+}$ mouse fibroblasts [41] were transfected, and the BLOCK-iT™ Fluorescent oligo (Invitrogen) was used to monitor the transfection efficiencies. At 3 days post-transfection, the cells were harvested for RNA isolation (see below) or processed for (immuno)fluorescence microscopy (see above).

RNA isolation and Northern blot analysis. Total RNA was isolated from cells using the Trizol reagent (Invitrogen). Total RNA was separated by electrophoresis through a 1% (w/v) denaturing agarose gel and transferred onto a nylon membrane. The amount of the mRNA coding for the β-subunit of FTase was detected using a 635-bp ^{32}P-labeled gene-specific DNA probe that was obtained by PCR (template: mouse liver cDNA [43]; primers: $FTbF_2$ and $FTbR_2$; Table 1). The blots were analyzed with a phosphorimaging device (Molecular Dynamics).

Antibodies. The polyclonal antiserum against $(His)_6$-$Gal4p_{AD}$ was raised in New Zealand White rabbits as previously described [35]. Animal care approval was granted by the local institutional ethics committee. The rabbit polyclonal antibodies against $(His)_6$-Pex13p, $(His)_6$-Pex14p, $(His)_6$-Pex19p, peroxisomal thiolase and GST were raised and employed as described elsewhere [31, 44, 45]. The rabbit anti-FTase antibody was obtained from Calbiochem, and the anti-farnesyl and secondary antibodies were purchased from Sigma.

Purification of recombinant proteins. Plasmids for recombinant protein expression were transformed into *E. coli* BL21(DE3) (T7-promoter) (Novagen) or *E. coli* Top10F' (non-T7 promoter) (Invitrogen). Bacterial lysates containing $(His)_6$-$HsPex5pRpA_{(1–624)}$, $(His)_6$-$HsPex13p_{(155–233)}$, $(His)_6$-$HsPex14p_{(1–377)}$, biotinylated RnDCR-AKL, biotinylated $HsPex13p_{(155–233)}$, biotinylated $HsPex14p_{(1–377)}$, or GST-HsPex19p were prepared essentially as described [46]. The $(His)_6$-tagged (or biotinylated) fusion proteins were affinity purified by employing Ni^{2+}-NTA (Qiagen) (or streptavidin; Pierce) agarose beads and ice-cold binding buffer B1 consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100, 10% (w/v) glycerol, and a protease inhibitor mixture (1 µg/ml aprotinin, 0.5 µg/ml leupeptin, 1 µg/ml α_2-macroglobulin, and 1 µg/ml chymostatin). Before use, the coated beads were washed five times with the same buffer. GST-HsPex19p was affinity purified by employing glutathione Sepharose 4B matrix (GE Healthcare) and ice-cold binding buffer B2 consisting of 50 mM Tris-HCl (pH 8.0), 0.5% (w/v) Triton X-100, and the protease inhibitor mixture. After five washes with 50 mM Tris-HCl (pH 8.0), GST-HsPex19p was selectively eluted from the affinity matrix with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). Before use, the eluted protein was dialyzed against the FTase dilution buffer (50 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 1 mM DTT).

***In vitro* farnesylation of HsPex19p.** Affinity-purified GST-HsPex19p (see above) was farnesylated *in vitro* by employing recombinant *S. cerevisiae* FTase (Sigma). Briefly, the standard reaction mixture contained the following components in a final volume of 30 µl: 33 mM

Tris-HCl (pH 7.5), 15 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 15 mM NaCl, 3.6 mM DTT, 2–13 µM farnesyl pyrophosphate (FPP) (Sigma), 300 nM [^{3}H]FPP (typically at 60 Ci/mmol) (ARC), 7.5 µM GST-HsPex19p, and 100 ng FTase. Note that the radiochemical purity of [^{3}H]FPP was at least 75% as judged by thin layer chromatography (silica gel 60; solvent: 60% 2-propanol/40% NH_4OH; data not shown). Assays were conducted at 33 °C for the times indicated. Following the incubation, 5 µl of each reaction was stopped by the addition of 1 ml of 37% (v/v) HCl:ethanol (1 : 9). After 15 min of incubation at room temperature, the protein precipitates were trapped on 25-mm GF/F filters (Whatman) and washed with ethanol (3 × 5 ml) and acetone (1 × 5 ml) by vacuum filtration. The amount of [^{3}H]farnesyl transferred to the GST-HsPex19p was measured by scintillation counting. 'No enzyme' reactions were performed for background subtractions (values ≤ 5% of the real signal observed). To visualize GST-HsPex19p by autoradiography, 15 µl of the reaction mixture was precipitated by the addition of 85 µl H_2O, 10 µl 0.15% (w/v) deoxycholate and 10 µl 72% (w/v) trichloroacetic acid (TCA). The pellet was washed with 1 ml acetone and subjected to SDS-PAGE. After electrophoresis, the gels were stained with Coomassie blue R-250, impregnated with 1 M sodium salicylate, dried and autoradiographed for 10 days at –80 °C with intensifying screens.

***In vitro* binding assay.** To perform pull-down assays with *in vitro* farnesylated Pex19p, 50 µl Ni^{2+}-NTA agarose beads coated with 1–10 µg of $(His)_6$-HsPex5pRpA$_{(1–624)}$, $(His)_6$-HsPex13p$_{(155–233)}$, or $(His)_6$-HsPex14p were resuspended in 100 µl of binding buffer B1 containing 2 µl nonfarnesylated (= 'no enzyme' reaction) or *in vitro* farnesylated GST-Pex19p (see above). After being rotated for 1 h at 4 °C, the microfuge tubes were subjected to a short spin and the nonbound fraction was transferred to another microfuge tube. The beads were washed five times with ice-cold binding buffer B1, and five times with wash buffer W1 (= binding buffer B1 minus glycerol). Bound proteins were eluted from the beads by adding 100 µl of elution buffer E1 (= wash buffer W1 supplemented with 250 mM imidazole pH 8.0). The total amount of GST-Pex19p in the bound and nonbound fractions was determined by the enzymatic detection of GST using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (for more details, see the 'GST detection module' instruction manual from GE Healthcare). To determine the distribution of farnesylated GST-Pex19p, incorporated [^{3}H]farnesyl was measured by scintillation counting after acid precipitation of the proteins (see above). Pull-down assays employed to study the interactions between biotinylated RnDCR-AKL, biotinylated HsPex13p$_{(155–233)}$, biotinylated HsPex14p$_{(1–377)}$, and HsPex19p variants expressed in CHO cells were basically performed as described elsewhere [46].

Results

Expression, purification and *in vitro* farnesylation of human Pex19p. We have previously shown that Pex19p$_{\Delta CAAX}$ has a strongly reduced binding affinity for Pex10p, Pex12p, and Pex13p in the yeast two-hybrid system [31]. As we have direct experimental evidence that this reduced binding is not the result of a lower expression level of Pex19p$_{\Delta CAAX}$ (I. Vastiau and M. Fransen, unpublished results), these observations suggest that farnesylation is an important determinant in the affinity of Pex19p for these proteins. To compare the affinity of farnesylated and nonfarnesylated Pex19p for PMPs, we endeavored to prepare farnesylated Pex19p *in vitro*. Therefore, purified Pex19p fused with glutathione S-transferase (GST-HsPex19p) was incubated with [^{3}H]FPP in the presence or absence of recombinant yeast FTase (Fig. 1a). Incorporation of the farnesyl group into GST-HsPex19p was observed only in the presence of FTase (Fig. 1b, c). The labeling efficiency could not be improved by changing the substrate concentration or buffer conditions (*e.g.* addition of $ZnCl_2$, reduction of DTT concentration, *etc*.) [47], the addition of rabbit reticulocyte lysate [48], the addition of new FTase after 30 min, or the use of $(His)_6$-Pex19p as the protein substrate (data not shown) (see discussion for possible explanations).

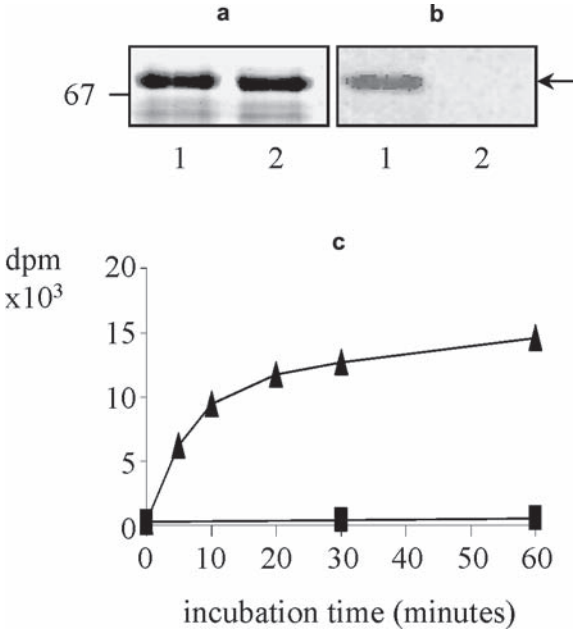

Figure 1. *In vitro* farnesylation of human Pex19p. Purified GST-HsPex19p was incubated with 2 µM of FPP in the presence (*a*, *b* lane 1; *c* triangle) or absence (*a*, *b* lane 2; *c* square) of recombinant yeast FTase. One-half of the reaction volume (see Materials and methods) was subjected to SDS-PAGE: (*a*) Coomassie blue R-250 staining; (*b*) autoradiography (exposure time: 10 days). The migration of the 67-kDa molecular mass marker is shown at the left. The arrow indicates GST-HsPex19p. (*c*) Time course of [^{3}H]farnesyl incorporation into GST-HsPex19p (each measurement represents one-sixth of the total reaction volume).

Maximal incorporation was obtained with 13 µM FPP (data not shown). However, further calculations revealed that even under this condition only ~35% of GST-HsPex19p was farnesylated. As attempts to separate farnesylated from nonfarnesylated GST-HsPex19p by reversed-phase chromatography, gel filtration chromatography, Triton X-114 phase separation, and immunoprecipitation with anti-farnesyl antibody were not successful (data not shown), the mixture was employed to perform *in vitro* binding experiments.

Farnesylation of human Pex19p does not alter its *in vitro* binding properties. To study the effect of farnesylation on the binding properties of Pex19p, a pull-down assay was used in which nonfarnesylated and *in vitro* farnesylated GST-HsPex19p were incubated with ligand-coated Ni^{2+}-NTA agarose beads. As yeast two-hybrid experiments have shown that deleting the *CAAX*-farnesylation motif of Pex19p decreased its binding to Pex10p, Pex11p*β*, Pex12p, and Pex13p nearly to background levels [31], we initially tried to express full-length $(His)_6$-tagged versions of these proteins in *E. coli*. Unfortunately, none of the proteins could be adequately expressed in soluble form (data not shown). However, we were able to express and purify sufficient quantities of tagged versions of HsPex13p$_{(155–233)}$, the Pex19p-binding domain of Pex13p [31], and HsPex14p, another Pex19p-interacting peroxisomal membrane protein [6]. Purified $(His)_6$-HsPex5Rp$_{(1–624)}$, a

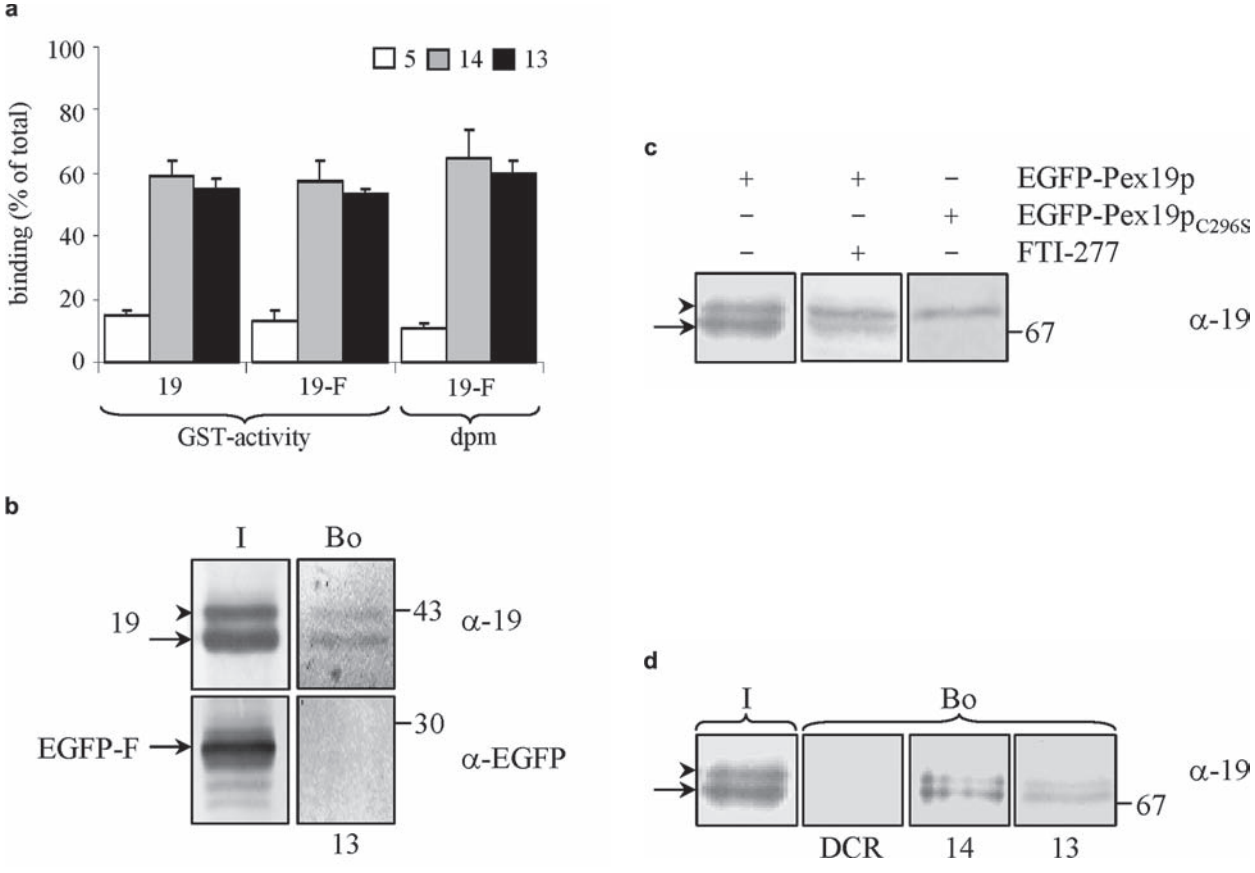

Figure 2. Effect of farnesylation on the binding properties of human Pex19p. (*a*) Ni^{2+}-NTA agarose beads were coated with $(His)_6$-HsPex5Rp$_{(1–624)}$ (5), $(His)_6$-HsPex14p$_{(1–377)}$ (14) or $(His)_6$-HsPex13p$_{(155–233)}$ (13). The coated beads were incubated with nonfarnesylated (= 'no enzyme' reaction) GST-Pex19p (19) or *in vitro* farnesylated GST-Pex19p (19-F) and processed as described in the Materials and methods. The total amount of GST-Pex19p in the bound and nonbound fractions was determined enzymatically using CDNB as a substrate (100% ≈ $\Delta OD_{340\,nm}$ = 0.1) for both 19 and 19-F. The distribution of farnesylated GST-Pex19p between the bound and nonbound fractions of 19-F was evaluated by scintillation counting (100% ≈ 2500 dpm). The values shown are the mean (± standard error) of at least two measurements. (*b*, *d*) Streptavidin beads coated with biotinylated RnDCR-AKL (DCR), HsPex14p$_{(1–377)}$ (14), or HsPex13p$_{(155–233)}$ (13) were incubated with the supernatant of a lysate of CHO cells (*b*) cotransfected with plasmids coding for HsPex19p and EGFP-F and grown in the presence of 2 µM FTI-277, or (*d*) transfected with a plasmid coding for EGFP-Pex19p. After thorough washing, the proteins bound to the coated streptavidin beads were separated by SDS-PAGE, transferred to nitrocellulose, and visualized using antibodies specific for Pex19p (*α*-19) or EGFP (*α*-EGFP). The signals represent 5% of the input fraction (I) or 20% of the bound fraction (Bo). (*c*) Immunoblot analysis of equal amounts of extracts from CHO cells transfected with plasmids coding for EGFP-Pex19p or EGFP-Pex19p$_{C296S}$ and grown in the absence or presence of 2 µM FTI-277. The arrows indicate EGFP or HsPex19p with 'wild-type' mobility, representing a prenylated (farnesylated in the absence of FTI-277, and farnesylated or geranylgeranylated in the presence of FTI-277) subform of Pex19p. The arrowheads mark nonprenylated HsPex19p. The migrations of the molecular mass markers (expressed in kilodaltons) are indicated.

non-peroxisomal Rab8b-interacting protein, or biotinylated RnDCR-AKL, a PTS1-containing peroxisomal matrix protein, were used as negative controls [35, 38, 49]. Quantitative measurements of GST-Pex19p in the bound and nonbound fractions indicated that equivalent portions of partially farnesylated and nonfarnesylated HsPex19p bound $Pex13p_{(155–233)}$ and Pex14p (Fig. 2a). As the *in vitro* farnesylated Pex19p has not undergone any of the post-prenylation processing steps, similar experiments were performed with CHO cell lysates containing *in vivo* prenylated and nonprenylated variants of Pex19p (see below). Again, no difference in affinity could be observed between the different forms of the protein (Fig. 2b, d). Although suggestive, these findings are not entirely conclusive because of obvious potential methodological limitations. For example, the employed recombinant epitope-tagged (truncated) proteins may not be properly folded. Therefore, we adopted an alternative *in vivo* approach.

The *CAAX* motif of human Pex19p is not essential to restore peroxisome biogenesis in Pex19p-deficient fibroblasts. In view of the conflicting results reported in the literature [5, 23, 25], we first reinvestigated whether or not a Pex19p-variant lacking the *CAAX* box could restore the formation of normal peroxisomes in Pex19p-deficient fibroblasts. These fibroblasts entirely lack morphologically recognizable peroxisomes when investigated by immunofluorescence (or direct fluorescence) analysis, and peroxisomal proteins are mislocalized to the cytoplasm or mitochondria and/or rapidly degraded [5, 23, 50]. Our results confirm the findings of Sacksteder et al. [5] and Mayerhofer et al. [25] that the *CAAX* box of Pex19p is not required for its biological function. That is, expression of EGFP-$Pex19p_{\Delta CAAX}$ in these cells restores Pex14p (Fig. 3, left column), PMP70 (data not shown), and DsRed-PTS1 (Fig. 3, right column) import into peroxisomes. In addition, no difference in complementation efficiency was observed between EGFP-$Pex19p_{\Delta CAAX}$ and EGFP-Pex19p (data not shown). However, immunoblot analyses of whole cell lysates from wild-type cells and Pex19p-deficient cells transiently transfected with the plasmid coding for EGFP-$Pex19p_{\Delta CAAX}$ revealed that the expression levels of the recombinant protein were at least 20-fold higher than those of the wild-type protein (data not shown). Note also that EGFP-$Pex19p_{\Delta CAAX}$ partially co-localizes with Pex14p and DsRed-PTS1 (Fig. 3). This observation indicates that farnesylation of Pex19p is not essential for its docking on the peroxisome membrane. Similar results were obtained with EGFP-$Pex19p_{C296S}$ (data not shown).

Targeting of proteins to peroxisomes does not require FTase. As restoration of *pex19* peroxisome assembly defects by EGFP-$HsPex19p_{\Delta CAAX}$ may be the result of overexpression, the experimental setup was modified such that the effect of farnesylation of endogenously expressed Pex19p on peroxisome biogenesis in wild-type cells could be studied. Mammalian cells contain two *CAAX* prenyltransferases: FTase and geranylgeranyltransferase 1 [51]. Each of these enzymes consists of two subunits, α and β, and the α-subunit is encoded by the same gene [51]. To selectively block FTase activity, we used FTI-277, a cell-permeable prodrug form of the highly potent and selective FTase inhibitor FTI-276, and Stealth™ RNAi oligonucleotides designed to specifically down-regulate the β-subunit of mouse FTase. As shown in Figure 4, peroxisomal targeting of Pex14p and DsRed-PTS1 was not affected in

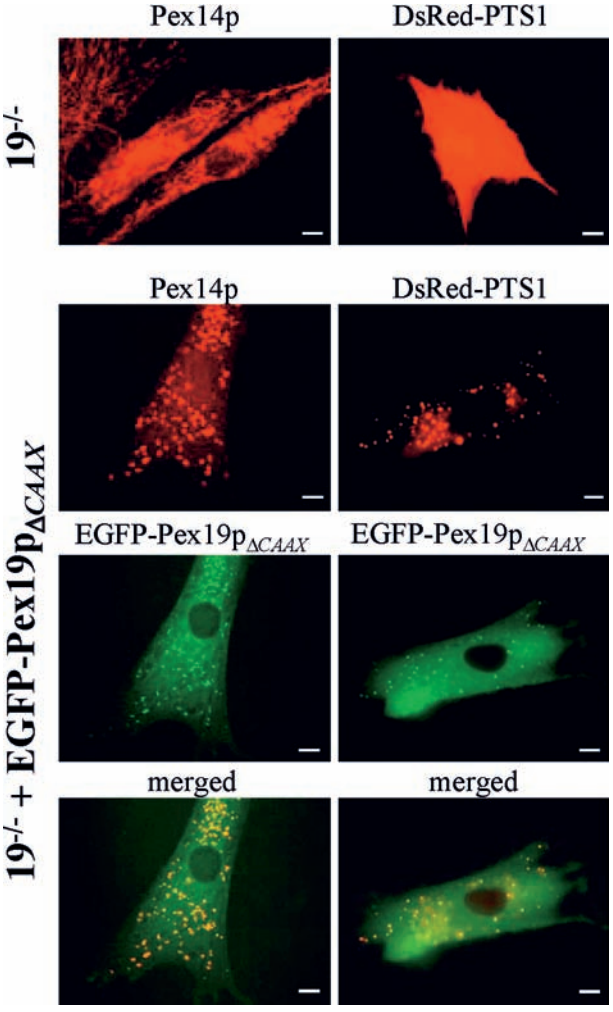

Figure 3. The prenylation motif of Pex19p is not essential to restore peroxisome biogenesis in Pex19p-deficient fibroblasts. Pex14p, a peroxisomal membrane protein, and DsRed-PTS1, a peroxisomal matrix marker protein, are mislocalized to the mitochondria and the cytoplasm, respectively, in Pex19p-deficient human fibroblasts ($19^{-/-}$). Transfection of the cells with a plasmid encoding EGFP-$Pex19p_{\Delta CAAX}$ ($19^{-/-}$ + EGFP-$Pex19p_{\Delta CAAX}$) and (immuno)fluorescence analysis of Pex14p and DsRed-PTS1 yielded, 5 days post-transfection, a punctate staining pattern indicating the reconstitution of functional peroxisomes. Note that EGFP-$Pex19p_{\Delta CAAX}$ displays a dual cytoplasmic/punctate distribution pattern. The punctate structures observed are peroxisomes, as illustrated by their colocalization with Pex14p and DsRed-PTS1. Scale bar represents 10 μm.

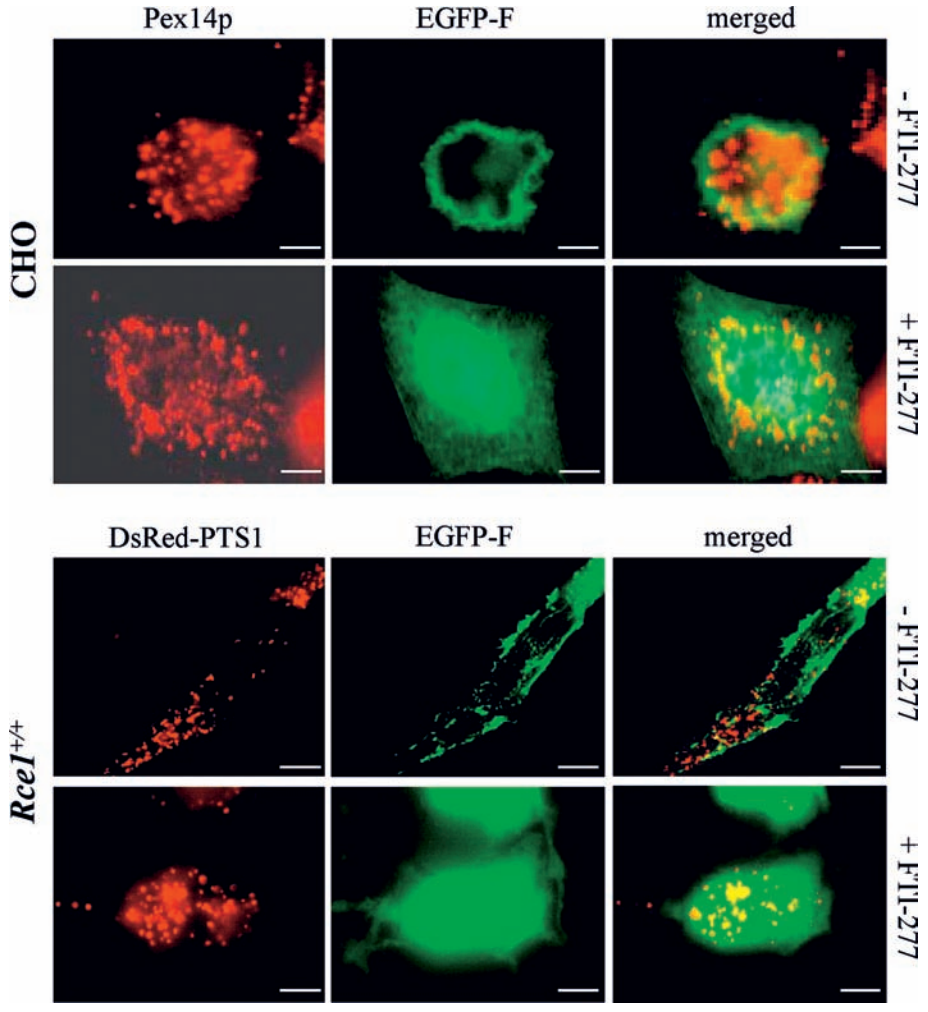

Figure 4. The FTase inhibitor FTI-277 does not inhibit protein targeting to peroxisomes. Transiently transfected CHO cells or *Rce1*$^{+/+}$ mouse fibroblasts expressing farnesylated EGFP (EGFP-F) and/or DsRed-PTS1 were grown in the absence (–) or presence (+) of 5 μM FTI-277. After 3 days, the cells were fixed and processed for direct fluorescence analysis or immunostaining with antibodies specific for endogenous Pex14p. Scale bar represents 10 μm.

mammalian cells grown in the presence of FTI-277. To confirm that the FTase activity was indeed inhibited under these conditions, the cells were (co-)transfected with a plasmid encoding EGFP-C-Ha-Ras$_{(170–189)}$ (abbreviated: EGFP-F). EGFP-F is a fluorescent protein containing the farnesylation signal from C-Ha-Ras [52], and it has been shown that farnesylation, followed by palmitoylation, directs the protein to the inner face of the plasma membrane [53]. As EGFP-F predominantly localized to the plasma membrane in mock-treated cells and in the cytoplasm and nucleus in FTI-277-treated cells (Fig. 4), our results clearly show that the drug entered the cells and that FTase activity – and thus farnesylation of Pex19p – is not essential for targeting proteins to peroxisomes. Post-transcriptional gene silencing of the β-subunit of FTase in mouse fibroblasts yielded essentially the same results. That is, down-regulation of the β-subunit mRNA by synthetic oligonucleotides did effect the subcellular localization of EGFP-F but not the peroxisomal targeting of Pex14p, DsRed-PTS1, and PMP70 (data not shown). Note that, from an experimental design standpoint, it is always possible to argue that if a cell contains peroxisomes and a drug that blocks a critical step for the formation of new peroxisomes is added, no effect will be seen. However, it has been reported that peroxisome turnover in mammals requires approximately 1.5 days [54, 55], and the cells were examined 3 days post-treatment. Also, previous experiments have already shown that, within this time span, (i) inhibition of Pex19p activity does result in a defect in PMP import [56], and (ii) peroxisomes are no longer discernible in CHO cells ectopically expressing Pex16p$_{(244–336)}$ [57]. As we presently lack antibodies of sufficient titer to detect the β-subunit of FTase in mouse fibroblasts (data not shown), we were unable to visualize the altered expression levels of the protein by immunoblot analysis. However, as farnesylation (and/or the associated proteolysis and carboxyl methylation events) causes a slight increase in the mobility of a protein in

SDS-polyacrylamide gels [58], we determined the electrophoretic mobility of HsPex19p after expression in control and FTI-277-treated mouse fibroblasts. As shown in Figure 5a (upper panel), a small but significant amount of HsPex19p with 'wild-type' mobility was observed in extracts prepared from FTI-277-treated fibroblasts. This observation suggests that, under the conditions employed, FTI-277 did not completely inhibit FTase activity. However, as (i) this conclusion is not in agreement with the observation that, under exactly the same conditions, the vast majority of EGFP-F is localized in the cytoplasm and nucleus (Fig. 4), (ii) FTase inhibitor treatments may lead to increased protein geranylgeranylation [59], and (iii) Prenylation Prediction Suite (http://mendel.imp.univie.ac.at/sat/PrepS), a recently developed software program [60], predicted that human Pex19p is a substrate for both FTase and GGTase (data not shown), we also investigated the electrophoretic mobility of GST-HsPex19p expressed in a wild-type and a *RAM1* (= gene coding for the β-subunit of FTase)-deletion strain. Again, although mutations in *RAM1* abolish FTase activity [61], a significant portion of HsPex19p with 'wild-type' mobility was observed in extracts from the Δ*ram1* strain (Fig. 5b). To exclude the possibility that this phenotype was a result of contamination of the original (haploid) *ram1* yeast strain with wild-type yeast cells, similar experiments were performed with diploid yeast cells homozygous or heterozygous for null alleles of *RAM1*. Again, essentially the same results were obtained (Fig. 5c). In this context, it is also interesting to note that it has already been demonstrated that mutations in RAM1 may result in crossprenylation of FTase substrates [62]. We also examined Pex19p prenylation following exposure to GGTI-298, a cell-permeable prodrug form of the GGTase I inhibitor GGTI-297, either alone or in combination with FTI-277. No effect was seen with GGTI-298 alone (Fig. 5a, lower panel). Co-treatment of cells with FTI-277 and GGTI-298 resulted in a much stronger inhibition of Pex19p processing than with FTI-277 alone (Fig. 5a, lower panel). A similar result was obtained for cells grown in the presence of 25 µM FTI-277 (data not shown). Summarized, these results indicate that the HsPex19p protein with 'wild-type' mobility in FTI-277-treated mouse fibroblasts and Δ*ram1* yeast cells is not farnesylated, but geranylgeranylated. Note that, even under conditions (*e.g.* 5 µM FTI-277 and 10 µM GGTI-298) where Pex19p was neither farnesylated nor geranylgeranylated, peroxisomal proteins displayed a correct subcellular localization (Fig. 6).

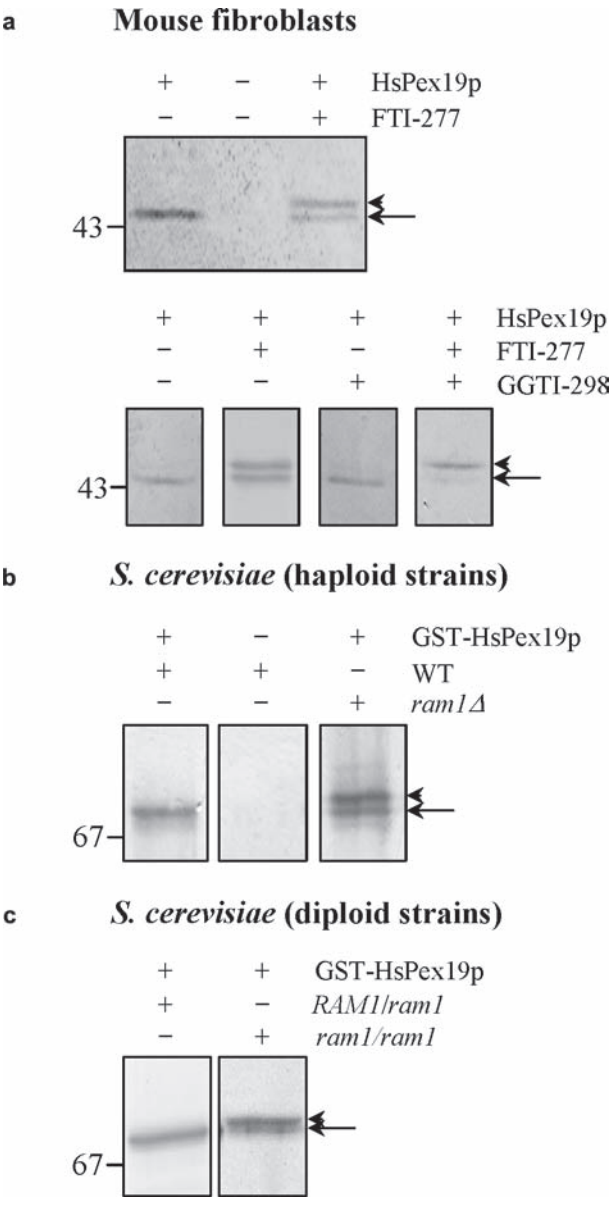

Figure 5. Electrophoretic mobility of HsPex19p. (*a*) Equal amounts of extracts from mouse fibroblasts expressing HsPex19p, treated or not with 5 µM FTI-277 and/or 10 µM GGTI-298, were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were then probed with an antiserum raised against HsPex19p. The arrows indicate HsPex19p with 'wild-type' mobility, representing a prenylated (farnesylated in the absence of FTI-277 or geranylgeranylated in the presence of FTI-277 alone) subform of Pex19p. The arrowheads mark nonprenylated HsPex19p. (*b*, *c*) Equal amounts of extracts from (*b*) haploid or (*c*) diploid yeast strains expressing GST-Pex19p were processed as described for the mouse fibroblasts. The arrows indicate HsPex19p with 'wild-type' mobility, representing a prenylated (farnesylated or geranylgeranylated in the presence or absence of RAM1 activity, respectively) subform of Pex19p. The arrowheads mark nonprenylated HsPex19p. The migration of relevant molecular mass markers (expressed in kDa) is shown at the left.

The post-farnesylation *CAAX*-processing steps are not required for proper localization of peroxisomal proteins. To investigate whether or not individual enzymes catalyzing the post-farnesylation steps are essential for peroxisome biogenesis, we determined the subcellular localization of peroxisomal proteins in mouse embryonic fibroblasts lacking Rce1 or Icmt. Rce1 and Icmt are responsible for the removal of the *–AAX* sequence and the carboxyl methylation of the newly exposed isoprenyl-

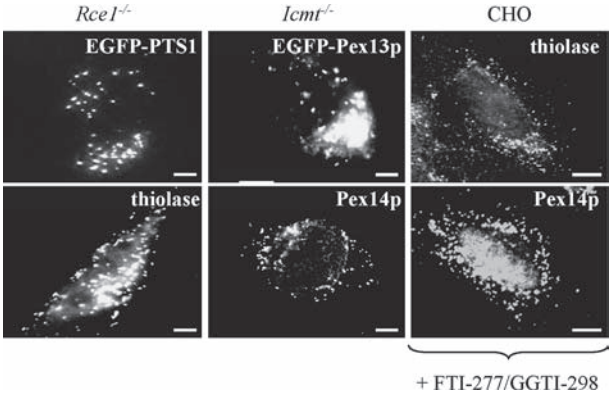

Figure 6. A disturbance of the (post-)farnesylation steps does not affect protein targeting to peroxisomes. CHO cells, grown in the presence of 5 µM FTI-277 and 10 µM GGTI-298, or mouse fibroblasts deficient in the endoprotease Rce1 (*Rce1*$^{-/-}$) or isoprenylcysteine carboxyl methyltransferase (*Icmt*$^{-/-}$) were transfected with a construct coding for EGFP-PTS1 or EGFP-Pex13p or were not transfected. The mouse fibroblasts and CHO cells were fixed after 24 and 72 h, respectively, and further processed for direct fluorescence or immunostained with antibodies specific for endogenous expressed peroxisomal thiolase or Pex14p. Scale bar represents 10 µm.

cysteine, respectively [40, 41]. Fluorescence microscopy analysis of both cell types showed a normal peroxisomal pattern of both endogenously (*e.g.* thiolase, Pex14p, and PMP70) and heterologously (*e.g.* DsRed-PTS1 and Pex13p-EGFP) expressed peroxisomal membrane and matrix proteins (Fig. 6; data not shown). In addition, β-oxidation of 2-methyl branched long chain fatty acids and α-oxidation of 3-methyl branched fatty acids, two peroxisomal processes, were not affected in these fibroblasts (P. P. Van Veldhoven, I. Vastiau and M. Fransen, unpublished results). These observations indicate that none of the post-farnesylation *CAAX*-processing steps are required for efficient functioning of the peroxisomal protein import machinery.

The farnesylation pathway is not essential for peroxisome biogenesis in *S. cerevisiae*. As our results indicated that farnesylation of Pex19p is not essential for the formation of functional peroxisomes in mammalian cells, we also investigated whether or not the farnesylation pathway is essential for Pex19p function in the yeast *S. cerevisiae*.

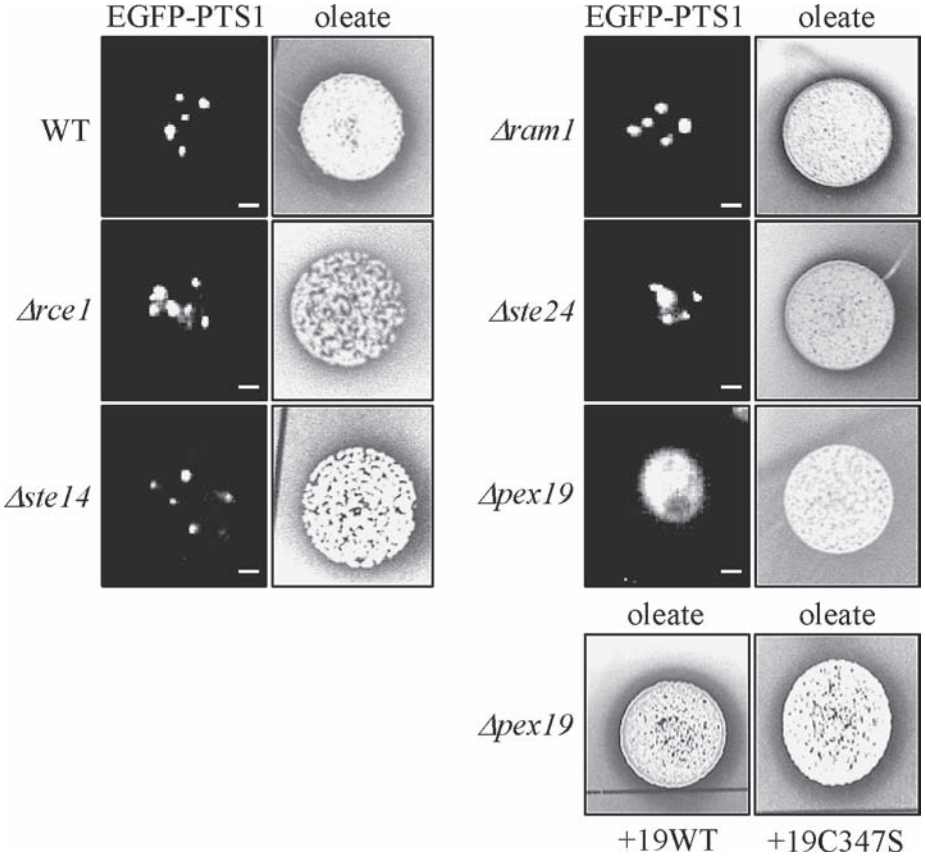

Figure 7. Farnesylation of Pex19p is not essential for peroxisome biogenesis in *S. cerevisiae*. Wild-type (WT) yeast cells (strain BY4741) or yeast cells deficient in the β-subunit of FTase (Δ*ram1*), the endoprotease Rce1p (Δ*rce1*), the endoprotease Ste24p (Δ*ste24*), isoprenylcysteine carboxyl methyltransferase Ste14p (Δ*ste14*), or Pex19p (Δ*pex19*) were transformed with a multicopy plasmid encoding EGFP-PTS1, the single-copy YCplac33-derivative encoding ScPex19p (19WT), or a YCplac33-derivative encoding ScPex19p$_{C347S}$ (19C347S). After 3 days of growth on (selective) medium, the cells were viewed under a fluorescence microscope or processed for growth on oleic acid medium and incubated for 5 additional days. Scale bar represents 1 µm.

We employed fluorescence microscopy to determine the subcellular localization of peroxisomal marker proteins in yeast open reading frame deletion strains deficient in *RAM1* (= gene encoding the *β*-subunit of FTase), *STE24* (= gene encoding a prenyl-dependent *CAAX* protease involved in a-factor maturation), *RCE1* (= gene encoding a second prenyl-dependent *CAAX* protease involved in Ras and a-factor maturation), or *STE14* (= gene encoding farnesylcysteine-carboxyl methyltransferase). These studies did not reveal any detectable peroxisomal sorting defect for EGFP-PTS1 (Fig. 7) or Pex11p-EGFP (data not shown). Next, we investigated whether or not (i) these deletion mutants contained functional peroxisomes, and (ii) $Pex19p_{C347S}$, a farnesylation deficient variant of Pex19p, was able to restore peroxisome biogenesis in a *pex19*Δ strain. Functional peroxisomes are essential for growth on oleic acid as the sole carbon source. To score consumption of the lipid, halo formation is routinely examined [63]. Here we employed a single-copy plasmid to express $Pex19p_{C347S}$ and wild-type Pex19p under control of the natural promoter in a *pex19*Δ strain, and determined the ability of the transformed cells to utilize oleic acid. As (i) yeast cells deficient in *RAM1*, *RCE1*, *STE24*, and *STE14* were able to consume oleate (Fig. 7), and (ii) genetic complementation of the *pex19*Δ deletion mutant with a plasmid encoding $Pex19p_{C347S}$ restored halo formation to wild-type levels (Fig. 7), farnesylation of *S. cerevisiae* Pex19p does not seem to be essential for its biological activity.

Discussion

At present, conflicting evidence exists regarding the requirement of farnesylation for Pex19p function [5, 21, 23–25, 31, 34, 64]. To reconcile previously published observations, we have compared the PMP-binding properties of nonfarnesylated and farnesylated Pex19p, reinvestigated whether or not the *CAAX*-farnesylation consensus motif is dispensable for Pex19p function, and determined which enzymes of the farnesylation pathway are required for efficient functioning of the peroxisomal protein import machinery.

In this study, we were able to confirm the findings of Matsuzono et al. [23] and Gloeckner et al. [34] that human Pex19p is a substrate for protein farnesylation *in vitro*. Somewhat unexpectedly, the efficiency of this process was rather low and could not be improved by changing the buffer conditions, the addition of new FTase after 30 min, or the use of $(His)_6$-HsPex19p instead of GST-HsPex19p. However, as the addition of fresh protein substrate promoted the total incorporation of the farnesyl group, it appears likely that the *CAAX* motif of the purified recombinant substrate protein is partially hidden or degraded. Also, as our FTI-277 studies indicate that most, if not all, HsPex19p is efficiently farnesylated *in vivo*, and it has been reported that the efficiency of protein prenylation in a cell-free system may differ from the efficiency *in vivo* [65], we cannot exclude the possibility that additional proteins or other factors are needed [66].

Previously, yeast two-hybrid experiments have shown that the *CAAX*-farnesylation motif of Pex19p is an important determinant in the affinity of Pex19p for Pex10p, Pex12p, and Pex13p [31]. Here we were unable to detect any difference in the PMP-binding properties of nonfarnesylated and farnesylated HsPex19p. This result is in agreement with other reports showing that nonfarnesylated HsPex19p interacts with ALDP, ALDPR, PMP34, PMP70, Pex3p, Pex12p, Pex13p, and Pex14p *in vitro* [5, 25, 31, 34, 46]. In addition, this study extends previous research by showing that the PMP-binding properties of nonfarnesylated and farnesylated HsPex19p are essentially the same. Note that, while this manuscript was in preparation, Matsuzono and Fujiki have also shown that the farnesylated and nonfarnesylated Pex19p behave indistinguishably with respect to their binding to Pex16p and Pex26p [67]. Why $HsPex19p_{\Delta CAAX}$ displays a strongly reduced affinity for Pex10p, Pex12p, and Pex13p in the yeast two-hybrid system, is currently not clear. One possibility may be that deletion of the C-terminal tetrapeptide of Pex19p changes the folding of the protein in such a manner that the binding to these PMPs is affected.

For a number of reasons (see above), our *in vitro* results may not completely reflect the *in vivo* situation. For example, other PMPs may behave differently from those tested. Note that we spent a considerable amount of time and effort to express full-length Pex10p, Pex11p*β*, Pex12p, and Pex13p in *E. coli*. However, as already mentioned above, none of these proteins could be adequately expressed in soluble form. On the other hand, we were able to obtain sufficient quantities of $HsPex12p_{(275–359)}$, the Pex19p-binding domain of HsPex12p [31]. However, as $HsPex12p_{(275–359)}$ did not bind to either (farnesylated and nonfarnesylated) Pex19p or to Pex5p, another known interaction partner ([6], and references therein), the bacterially expressed protein is most likely not correctly folded (data not shown). To address this issue more directly and completely, we adopted two alternative *in vivo* approaches: (i) we reinvestigated whether or not Pex19p variants lacking a functional *CAAX* box were able to restore the formation of functional peroxisomes in Pex19p-deficient fibroblasts and yeast cells; and (ii) we studied the subcellular localization of peroxisomal membrane and matrix proteins in mammalian and yeast cells displaying defects in one of the enzymatic steps of the *CAAX* protein maturation cascade.

With respect to the complementation studies of Pex19p-deficient fibroblasts, our results show that, under conditions of overexpression, farnesylation of Pex19p is not essential to restore the formation of (protein) import-

competent peroxisomes of normal appearance. These outcomes are in agreement with those reported by Sacksteder et al. [5]. Also, the observation that a small but significant part of the nonfarnesylated Pex19p population is associated with the peroxisome membrane, counters the hypothesis that farnesylation is required for peroxisomal localization of Pex19p [23]. Perhaps this is not surprising given that farnesylated proteins frequently require palmitoylation for membrane binding [68], and human Pex19p does not contain any consensus palmitoylation or myristoylation sites (data not shown). To avoid misinterpretation of results due to overexpression of HsPex19p$_{\Delta CAAX}$ (or HsPex19p$_{C296S}$), we investigated whether or not ScPex19p$_{C347S}$, expressed from a single-copy plasmid under control of the natural promoter, could complement peroxisome function in a *pex19*Δ yeast strain. No significant differences could be detected between ScPex19p$_{C347S}$ and ScPex19p for growth on oleic acid-containing medium. This observation suggests that farnesylation of Pex19p is not essential for peroxisome biogenesis in yeast. However, similar studies performed by Götte et al. [21] demonstrated that the expression of nonfarnesylated Pex19p in *pex19*Δ cells resulted in only partial complementation. Also, by employing subcellular fractionation techniques, these authors demonstrated that a portion of the peroxisomal proteins in these cells was mislocalized. Unfortunately, as we lack antibodies against peroxisomal marker proteins from *S. cerevisiae*, we could not directly confirm this result.

Finally, peroxisome morphology, peroxisomal protein import, and/or peroxisomal metabolism appeared similar in wild-type cells and fibroblasts lacking FTase, Rce1, or Icmt activities. Also, yeast strains deficient in one of these enzymes of the farnesylation pathway still contained functional peroxisomes. Interestingly, a small but significant portion of Pex19p is alternatively geranylgeranylated when farnesylation is inhibited. Treatment with GGTI-298 alone had no effect on the electrophoretic mobility of Pex19p. This observation indicates that Pex19p is normally not geranylgeranylated. The phenomenon known as 'cross-prenylation', which has already been reported for other proteins [69], may keep a protein biologically active and fully capable of functioning. However, as (i) variants of Pex19p lacking a functional prenylation motif were able to restore peroxisome biogenesis in human and yeast cells deficient in this peroxin, and (ii) peroxisomal targeting of membrane and matrix proteins was not affected in mammalian cells grown under conditions in which Pex19p was not post-translationally modified, prenylation of Pex19p is not essential for peroxisome biogenesis in these cells. Currently, our results do not exclude the possibility that, under certain conditions, farnesylation of Pex19p might affect the stability of the protein. However, our observation that EGFP-Pex19p and EGFP-Pex19p$_{C296S}$ are equally efficient in complementing peroxisome biogenesis in a Pex19p-deficient cell line (data not shown) is not in favor of this hypothesis.

In summary, our observations indicate that the *CAAX* box-mediated processing steps of Pex19p are dispensable for peroxisome biogenesis in mammals and yeast. This conclusion is in full agreement with the results reported for *P. pastoris* and *Y. lipolytica* Pex19p [24, 64], and further supported by the recent observation that the farnesylation motif of Pex19p is not evolutionarily conserved [26, 27].

Acknowledgements. We thank Dr. S. Terlecky (Wayne State University School of Medicine, Michigan) for critically reading the manuscript, Dr. B. Distel (University of Amsterdam, The Netherlands) for the pEW173 plasmid, Dr. A. Hartig (University of Vienna, Austria) for the pJR233 plasmid, Dr. G. Stier (EMBL Heidelberg, Germany) for the pETM11 vector, Dr. J. Thevelein (K.U. Leuven, Belgium) for the YCplac33 plasmid, Gerd Van der Hoeven (K.U. Leuven, Belgium) for FPP analysis, and Kristien De Greef (K.U. Leuven, Belgium) for cultivation of the mouse fibroblasts. This work was supported by grants from the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen (Onderzoeksproject G.0237.04), the Flemish government (Geconcerteerde Onderzoeksacties GOA/2004/08), and the FP6 European Union Project 'Peroxisome' (LSHG-CT-2004-512018).

1 Wanders, R. J. (2004) Peroxisomes, lipid metabolism, and peroxisomal disorders. Mol. Genet. Metab. 83, 16–27.
2 Moyersoen, J., Choe, J., Fan, E., Hol, W. G. and Michels, P. A. (2004) Biogenesis of peroxisomes and glycosomes: trypanosomatid glycosome assembly is a promising new drug target. FEMS Microbiol. Rev. 28, 603–643.
3 Distel, B., Erdmann, R., Gould, S. J., Blobel, G., Crane, D. I., Cregg, J. M., Dodt, G., Fujiki, Y., Goodman, J. M., Just, W. W., Kiel, J. A., Kunau, W. H., Lazarow, P. B., Mannaerts, G. P., Moser, H. W., Osumi, T., Rachubinski, R. A., Roscher, A., Subramani, S., Tabak, H. F., Tsukamoto, T., Valle, D., Van der Klei, I., Van Veldhoven, P. P. and Veenhuis, M. (1996) A unified nomenclature for peroxisome biogenesis factors. J. Cell Biol. 135, 1–3.
4 Snyder, W. B., Koller, A., Choy, A. J. and Subramani, S. (2000) The peroxin Pex19p interacts with multiple, integral membrane proteins at the peroxisomal membrane. J. Cell Biol. 149, 1171–1178.
5 Sacksteder, K. A., Jones, J. M., South, S. T., Li, X., Liu, Y. and Gould, S. J. (2000) PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. J. Cell Biol. 148, 931–944.
6 Fransen, M., Brees, C., Ghys, K., Amery, L., Mannaerts, G. P., Ladant, D. and Van Veldhoven, P. P. (2002) Analysis of mammalian peroxin interactions using a non-transcription-based bacterial two-hybrid assay. Mol. Cell. Proteomics 1, 243–252.
7 Eckert, J. H. and Johnsson, N. (2003) Pex10p links the ubiquitin-conjugating enzyme Pex4p to the protein import machinery of the peroxisome. J. Cell Sci. 116, 3623–3634.
8 Terlecky, S. R. and Fransen, M. (2000) How peroxisomes arise. Traffic 1, 465–473.
9 Brown, L. A. and Baker, A. (2003) Peroxisome biogenesis and the role of protein import. J. Cell. Mol. Med. 7, 388–400.
10 Hazra, P. P., Suriapranata, I., Snyder, W. B. and Subramani, S. (2002) Peroxisome remnants in pex3delta cells and the requirement of Pex3p for interactions between the peroxisomal docking and translocation subcomplexes. Traffic 3, 560–574.
11 Agne, B., Meindl, N. M., Niederhoff, K., Einwachter, H., Rehling, P., Sickmann, A., Meyer, H. E., Girzalsky, W. and Kunau,

W. H. (2003) Pex8p: an intraperoxisomal organizer of the peroxisomal import machinery. Mol. Cell. 11, 635–646.
12 Nooren, I. M. and Thornton, J. M. (2003) Diversity of protein-protein interactions. EMBO J. 22, 3486–3492.
13 Purdue, P. E. and Lazarow, P. B. (2001) Peroxisome biogenesis. Annu. Rev. Cell Dev. Biol. 17, 701–752.
14 Platta, H. W., Girzalsky, W. and Erdmann, R. (2004) Ubiquitination of the peroxisomal import receptor Pex5p. Biochem. J. 384, 37–45.
15 Kragt, A., Voorn-Brouwer, T., van den Berg, M. and Distel, B. (2005) The *Saccharomyces cerevisiae* peroxisomal import receptor Pex5p is monoubiquitinated in wild-type cells. J. Biol. Chem. 280, 7867–7874.
16 Kiel, J. A., Emmrich, K., Meyer, H. E. and Kunau, W. H. (2005) Ubiquitination of the peroxisomal targeting signal type 1 receptor, Pex5p, suggests the presence of a quality control mechanism during peroxisomal matrix protein import. J. Biol. Chem. 280, 1921–1930.
17 Léon, S., Zhang, L., McDonald, W. H., Yates, J., Cregg, J. M. and Subramani, S. (2006) Dynamics of the peroxisomal import cycle of PpPex20p: ubiquitin-dependent localization and regulation. J. Cell Biol. 172, 67–78.
18 Komori, M., Kiel, J. A. and Veenhuis, M. (1999) The peroxisomal membrane protein Pex14p of *Hansenula polymorpha* is phosphorylated *in vivo*. FEBS Lett. 457, 397–399.
19 Johnson, M. A., Snyder, W. B., Cereghino, J. L., Veenhuis, M., Subramani, S. and Cregg, J. M. (2001) *Pichia pastoris* Pex14p, a phosphorylated peroxisomal membrane protein, is part of a PTS-receptor docking complex and interacts with many peroxins. Yeast 18, 621–641.
20 Elgersma, Y., Kwast, L., van den Berg, M., Snyder, W. B., Distel, B., Subramani, S. and Tabak, H. F. (1997) Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S. cerevisiae*, causes proliferation of the endoplasmic reticulum membrane. EMBO J. 16, 7326–7341.
21 Götte, K., Girzalsky, W., Linkert, M., Baumgart, E., Kammerer, S., Kunau, W. H. and Erdmann, R. (1998) Pex19p, a farnesylated protein essential for peroxisome biogenesis. Mol. Cell. Biol. 18, 616–628.
22 Kammerer, S., Arnold, N., Gutensohn, W., Mewes, H. W., Kunau, W. H., Hofler, G., Roscher, A. A. and Braun, A. (1997) Genomic organization and molecular characterization of a gene encoding HsPXF, a human peroxisomal farnesylated protein. Genomics 45, 200–210.
23 Matsuzono, Y., Kinoshita, N., Tamura, S., Shimozawa, N., Hamasaki, M., Ghaedi, K., Wanders, R. J., Suzuki, Y., Kondo, N. and Fujiki, Y. (1999) Human PEX19: cDNA cloning by functional complementation, mutation analysis in a patient with Zellweger syndrome, and potential role in peroxisomal membrane assembly. Proc. Natl. Acad. Sci. USA 96, 2116–2121.
24 Snyder, W. B., Faber, K. N., Wenzel, T. J., Koller, A., Luers, G. H., Rangell, L., Keller, G. A. and Subramani, S. (1999) Pex19p interacts with Pex3p and Pex10p and is essential for peroxisome biogenesis in *Pichia pastoris.* Mol. Biol. Cell 10, 1745–1761.
25 Mayerhofer, P. U., Kattenfeld, T., Roscher, A. A. and Muntau, A. C. (2002) Two splice variants of human PEX19 exhibit distinct functions in peroxisomal assembly. Biochem. Biophys. Res. Commun. 291, 1180–1186.
26 Banerjee, S. K., Kessler, P. S., Saveria, T. and Parsons, M. (2005) Identification of trypanosomatid PEX19: functional characterization reveals impact on cell growth and glycosome size and number. Mol. Biochem. Parasitol. 142, 47–55.
27 Yernaux, C., Fransen, M., Brees, C., Lorenzen, S. and Michels, P. A. M. (2006) *Trypanosoma brucei* glycosomal ABC transporters: Identification and membrane targeting. Mol. Membr. Biol. 23, 157–172.
28 Zhang, F. L. and Casey, P. J. (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. 65, 241–269.
29 Magee, T. and Marshall, C. (1999) New insights into the interaction of Ras with the plasma membrane. Cell 98, 9–12.
30 Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E. and Philips, M. R. (1999) Endomembrane trafficking of Ras: the *CAAX* motif targets proteins to the ER and Golgi. Cell 98, 69–80.
31 Fransen, M., Wylin, T., Brees, C., Mannaerts, G. P. and Van Veldhoven, P. P. (2001) Human Pex19p binds peroxisomal integral membrane proteins at regions distinct from their sorting sequences. Mol. Cell. Biol. 21, 4413–4424.
32 Fang, Y., Morrell, J. C., Jones, J. M. and Gould, S. J. (2004) PEX3 functions as a PEX19 docking factor in the import of class I peroxisomal membrane proteins. J. Cell Biol. 164, 863–775.
33 Fransen, M., Vastiau, I., Brees, C., Brys, V., Mannaerts, G. P. and Van Veldhoven, P. P. (2005) Analysis of human Pex19p's domain structure by pentapeptide scanning mutagenesis. J. Mol. Biol. 346, 1275–1286.
34 Gloeckner, C. J., Mayerhofer, P. U., Landgraf, P., Muntau, A. C., Holzinger, A., Gerber, J. K., Kammerer, S., Adamski, J. and Roscher, A. A. (2000) Human adrenoleukodystrophy protein and related peroxisomal ABC transporters interact with the peroxisomal assembly protein PEX19p. Biochem. Biophys. Res. Commun. 271, 144–150.
35 Amery, L., Sano, H., Mannaerts, G. P., Snider, J., Van Looy, J., Fransen, M. and Van Veldhoven, P. P. (2001) Identification of PEX5p-related novel peroxisome-targeting signal 1 (PTS1)-binding proteins in mammals. Biochem. J. 357, 635–646.
36 Ghys, K., Fransen, M., Mannaerts, G. P. and Van Veldhoven, P. P. (2002) Functional studies on human Pex7p: subcellular localization and interaction with proteins containing a peroxisome-targeting signal type 2 and other peroxins. Biochem. J. 365, 41–50.
37 Brocard, C., Lametschwandtner, G., Koudelka, R. and Hartig, A. (1997) Pex14p is a member of the protein linkage map of Pex5p. EMBO J. 16, 5491–5500.
38 Fransen, M., Van Veldhoven, P. P. and Subramani, S. (1999) Identification of peroxisomal proteins by using M13 phage protein VI phage display: molecular evidence that mammalian peroxisomes contain a 2,4-dienoyl-CoA reductase. Biochem. J. 340, 561–568.
39 Fransen, M., Terlecky, S. R. and Subramani, S. (1998) Identification of a human PTS1 receptor docking protein directly required for peroxisomal protein import. Proc. Natl. Acad. Sci. USA 95, 8087–8092.
40 Bergo, M. O., Leung, G. K., Ambroziak, P., Otto, J. C., Casey, P. J., Gomes, A. Q., Seabra, M. C. and Young, S. G. (2001) Isoprenylcysteine carboxyl methyltransferase deficiency in mice. J. Biol. Chem. 276, 5841–5845.
41 Bergo, M. O., Ambroziak, P., Gregory, C., George, A., Otto, J. C., Kim, E., Nagase, H., Casey, P. J., Balmain, A. and Young, S. G. (2002) Absence of the *CAAX* endoprotease Rce1: effects on cell growth and transformation. Mol. Cell Biol. 22, 171–181.
42 Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B. and Behr, J. P. (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. Proc. Natl. Acad. Sci. USA 92, 7297–7301.
43 Huyghe, S., Casteels, M., Janssen, A., Meulders, L., Mannaerts, G. P., Declercq, P. E., Van Veldhoven, P. P. and Baes, M. (2001) Prenatal and postnatal development of peroxisomal lipid-metabolizing pathways in the mouse. Biochem. J. 353, 673–680.
44 Amery, L., Fransen, M., De Nys, K., Mannaerts, G. P. and Van Veldhoven, P. P. (2000) Mitochondrial and peroxisomal targeting of 2-methylacyl-CoA racemase in humans. J. Lipid Res. 41, 1752–1759.

45 Antonenkov, V. D., Croes, K., Waelkens, E., Van Veldhoven, P. P. and Mannaerts, G. P. (2000) Identification, purification and characterization of an acetoacetyl-CoA thiolase from rat liver peroxisomes. Eur. J. Biochem. 267, 2981–2990.
46 Fransen, M., Vastiau, I., Brees, C., Brys, V., Mannaerts, G. P. and Van Veldhoven, P. P. (2004) Potential role for Pex19p in assembly of PTS-receptor docking complexes. J. Biol. Chem. 279, 12615–12624.
47 Hightower, K. E., Casey, P. J. and Fierke, C. A. (2001) Farnesylation of nonpeptidic thiol compounds by protein farnesyltransferase. Biochemistry 40, 1002–1010.
48 Kanazawa, M., Terada, K., Kato, S. and Mori, M. (1997) HSDJ, a human homologue of DnaJ, is farnesylated and is involved in protein import into mitochondria. J. Biochem. 121, 890–895.
49 Chen, S., Liang, M. C., Chia, J. N., Ngsee, J. K. and Ting, A. E. (2001) Rab8b and its interacting partner TRIP8b are involved in regulated secretion in AtT20 cells. J. Biol. Chem. 276, 13209–13216.
50 Muntau, A. C., Roscher, A. A., Kunau, W. H. and Dodt, G. (2003) The interaction between human PEX3 and PEX19 characterized by fluorescence resonance energy transfer (FRET) analysis. Eur. J. Cell. Biol. 82, 333–342.
51 Maurer-Stroh, S., Washietl, S. and Eisenhaber, F. (2003) Protein prenyltransferases. Genome Biol. 4, 212.1–212.9.
52 Hancock, J. F., Paterson, H. and Marshall, C. J. (1990) A polybasic domain or palmitoylation is required in addition to the *CAAX* motif to localize p21ras to the plasma membrane. Cell 63, 133–139.
53 Jiang, W. and Hunter, T. (1998) Analysis of cell-cycle profiles in transfected cells using a membrane-targeted GFP. Biotechniques 24, 349–350, 352, 354.
54 Poole, B., Leighton, F. and De Duve, C. (1969) The synthesis and turnover of rat liver peroxisomes. II. Turnover of peroxisome proteins. J. Cell Biol. 41, 536–546.
55 Sartori, C., Stefanini, S., Cimini, A., Di Giulio, A. and Ceru, M. P. (1992) Liver peroxisomes in newborns from clofibrate-treated rats. II. A biochemical study of the recovery period. Biol. Cell 74, 315–324.
56 Jones, J. M., Morrell, J. C. and Gould, S. J. (2004) PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. J. Cell Biol. 164, 57–67.
57 Honsho, M., Hiroshige, T. and Fujiki, Y. (2002) The membrane biogenesis peroxin Pex16p: topogenesis and functional roles in peroxisomal membrane assembly. J. Biol. Chem. 277, 44513–44524.
58 Caplan, A. J., Tsai, J., Casey, P. J. and Douglas, M. G. (1992) Farnesylation of YDJ1p is required for function at elevated growth temperatures in *Saccharomyces cerevisiae.* J. Biol. Chem. 267, 18890–18895.
59 Prendergast, G. C. and Oliff, A. (2000) Farnesyltransferase inhibitors: antineoplastic properties, mechanisms of action, and clinical prospects. Semin. Cancer Biol. 10, 443–452.
60 Maurer-Stroh, S. and Eisenhaber, F. (2005) Refinement and prediction of protein prenylation motifs. Genome Biol. 6, R55.
61 He, B., Chen, P., Chen, S. Y., Vancura, K. L., Michaelis, S. and Powers, S. (1991) *RAM2*, an essential gene of yeast, and *RAM1* encode the two polypeptide components of the farnesyltransferase that prenylates a-factor and Ras proteins. Proc. Natl. Acad. Sci. USA 88, 11373–11377.
62 Trueblood, C. E., Ohya, Y. and Rine, J. (1993) Genetic evidence for *in vivo* cross-specificity of the CaaX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase-I in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 13, 4260–4275.
63 Erdmann, R., Veenhuis, M., Mertens, D. and Kunau, W. H. (1989) Isolation of peroxisome-deficient mutants of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 86, 5419–5423.
64 Lambkin, G. R. and Rachubinski, R. A. (2001) *Yarrowia lipolytica* cells mutant for the peroxisomal peroxin Pex19p contain structures resembling wild-type peroxisomes. Mol. Biol. Cell 12, 3353–3364.
65 Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E. and Der, C. J. (1992) Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc. Natl. Acad. Sci. USA 89, 6403–6407.
66 Seabra, M. C., Mules, E. H. and Hume, A. N. (2002) Rab GTPases, intracellular traffic and disease. Trends Mol. Med. 8, 23–30.
67 Matsuzono, Y. and Fujiki, Y. (2006) *In vitro* transport of membrane proteins to peroxisomes by shuttling receptor Pex19p. J. Biol. Chem. 281, 36–42.
68 Gelb, M. H. (1997) Protein prenylation, *et cetera*: signal transduction in two dimensions. Science 275, 1750–1751.
69 Rowell, C. A., Kowalczyk, J. J., Lewis, M. D. and Garcia, A. M. (1997) Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras *in vivo*. J. Biol. Chem. 272, 14093–14097.