University of Alberta

The Peroxin Pex34p Functions with the Pex11 Family of Proteins to Regulate the Peroxisome Population in the Yeast *Saccharomyces cerevisiae*

by

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Abstract

Peroxisomes are highly dynamic organelles that change in size and number in response to both intra- and extracellular cues. Peroxisome growth and division are controlled by the import of matrix proteins and by a specialized divisional machinery that includes peroxisome-specific factors, such as members of the Pex11 protein family, and general organelle divisional factors, such as the dynamin-related protein Vps1p. Here we show that Pex34p is a peroxisomal integral membrane protein that acts independently and together with the Pex11 protein family members Pex11p, Pex25p and Pex27p to control the peroxisome populations of cells under conditions of both peroxisome proliferation and constitutive peroxisome division. Pex34p can act as a positive effector of peroxisome division but requires the Pex11 family proteins. Our addition of Pex34p to the repertoire of proteins involved in regulating peroxisome division emphasizes the necessity of cells to strictly regulate their peroxisome populations.

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LIST OF ABBREVIATIONS

20KgP	pellet obtained from centrifugation at 20,000 x *g*
20KgS	supernatant obtained from centrifugation at 20,000 x *g*
DRP	dynamin-related protein
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
g	gravitational force
GFP	green fluorescent protein
HRP	horseradish peroxidase
mRFP	monomeric red fluorescent protein
PTS	peroxisome targeting signal
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBD	peroxisome biogenesis disorder
PCR	polymerase chain reaction
PEX#	wild-type gene encoding Pex#p
pex#	mutant *PEX#* gene
PMP	peroxisomal membrane protein
PNS	postnuclear supernatant
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
X-gal	5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside
TAP	tandem-affinity purification

CHAPTER 1

INTRODUCTION

1.1 Introduction to peroxisomes

Eukaryotic cells have an advantage over prokaryotic cells by having membrane-bound organelles that provide optimized microenvironments. Peroxisomes are single membrane-bound organelles specialized for a variety of metabolic functions. They were defined by Christian de Duve and colleagues (de Duve, 1965; de Duve and Baudhuin, 1966) as organelles containing an oxidase, which produces hydrogen peroxide (H_2O_2), and catalase, which degrades H_2O_2. Peroxisomes have been identified in nearly all free living eukaryotes. The term peroxisome has also been applied to the glyoxysome of plants (Breidenbach and Beevers, 1967), the Woronin bodies of filamentous fungi (Markham and Collinge, 1987) and the glycosomes of Trypanosomatids (Opperdoes and Borst, 1977).

Two widely conserved functions of peroxisomes are the β-oxidation of fatty acids and the metabolism of H_2O_2. Other metabolic pathways found in the peroxisomes include the α-oxidation of branched-chain fatty acids, the catabolism of purines and the biosynthesis of bile acids and plasmalogens (for reviews, see Wanders and Waterham, 2006; Schrader and Fahimi, 2008). These pathways are dependent on the import of more than 50 matrix proteins, which are synthesized on free polysomes and imported through recognition of two unique peroxisome targeting signals (PTSs) called PTS1 and PTS2 (Gould *et al*., 1989; Swinkels *et al*., 1991; Rachubinski and Subramani, 1995; Mukal and Fujiki, 2006). The import of PTS1- and PTS2-containing proteins relies on the cytosolic receptors Pex5 and Pex7, respectively, along with a core import complex made up of integral and peripheral membrane proteins on the peroxisomal membrane that

facilitate the docking of Pex5- and Pex7-bound proteins, the formation of an import-competent pore in the peroxisomal membrane and the subsequent cycling and release of the import receptor back to the cytosol (Lazarow, 2006; Mast *et al*., 2010; Rucktaschel *et al*., 2011).

1.2 Peroxisome biogenesis disorders

Peroxisomes are essential for normal human development and physiology, as evidenced by the lethality of a spectrum of human diseases collectively known as the peroxisome biogenesis disorders (PBDs) (Steinberg *et al.*, 2006; Schrader and Fahimi, 2008). The PBDs are the result of mutations in one of 13 PEX genes and encompass defects in peroxisome biogenesis as well as impaired import of peroxisomal matrix proteins. The most common PBD, Zellweger syndrome, has an incidence rate of 1:50,000 live births and often results in death within the first year of life. Symptoms include facial-cranial malformation, severe muscular hypotonia, severe growth defects and developmental delays. Clinical diagnosis of PBD patients includes elevated levels of very-long chain fatty acids, the presence of the immature, non-cleaved form of peroxisomal 3-ketoacyl-CoA thiolase, build-up of phytanic acid and reduced levels of plasmalogens. To date, care of patients suffering from PBDs is palliative in nature. A better understanding of the causes of the PBDs has been a driving force behind the identification and characterization of the *PEX* genes involved in peroxisome biogenesis. To date, 34 *PEX* genes in a number of different organisms have been identified that are involved in the targeting and import of peroxisomal proteins, the formation of the

peroxisome membrane, and the control of peroxisome size and abundance (Schrader and Fahimi, 2008; Managadze *et al.*, 2010; Wolfe *et al.*, 2010; Tower *et al*., 2011).

1.3 Regulating the peroxisome population

Peroxisomes are dynamic organelles that respond to a variety of intra- and extracellular stimuli. Peroxisome size and abundance are controlled by multiple pathways (for reviews, see Yan *et al.*, 2005; Fagarasanu *et al.*, 2007; Tabak *et al.*, 2008; Hettema and Motley, 2009; Mast *et al.*, 2010; Saraya *et al.*, 2010). One pathway involves the response of cells to specific environmental or metabolic cues, such as growth of yeast on a non-fermentable fatty acid carbon source. This leads to the "induction" or up-regulation of the expression of genes encoding peroxisomal proteins and rapid expansion of the peroxisomal compartment through increases in both the number of peroxisomes (*i.e.*, peroxisome proliferation) and their sizes. In this manner, a cell can regulate its peroxisome population to induce a compartmental expansion under growth conditions in which peroxisome function is essential.

A second pathway termed peroxisome "constitutive division" functions to maintain the peroxisome population in both the mother cell and the newly forming bud during cell division. The peroxisome population doubles during the cell cycle independently of peroxisome-proliferating stimuli so that essentially equal numbers of peroxisomes can be maintained in the mother cell and apportioned to the daughter cell. In the yeast *Saccharomyces cerevisiae,*

peroxisomes that have doubled in number before cell division are equally partitioned between mother cell and bud through the interplay of Inp2p, the peroxisome-specific receptor for the myosin mediating bud-directed peroxisome transport (Fagarasanu *et al.*, 2006), and Inp1p, which acts in anchoring peroxisomes in both mother cell and bud (Fagarasanu *et al.*, 2005). The third pathway involves the *de novo* formation of peroxisomes from the endoplasmic reticulum (ER). Cells lacking peroxisomes or their remnants have the ability to reform functional peroxisomes from the ER (Hoepfner *et al.*, 2005; Tam *et al.*, 2005).

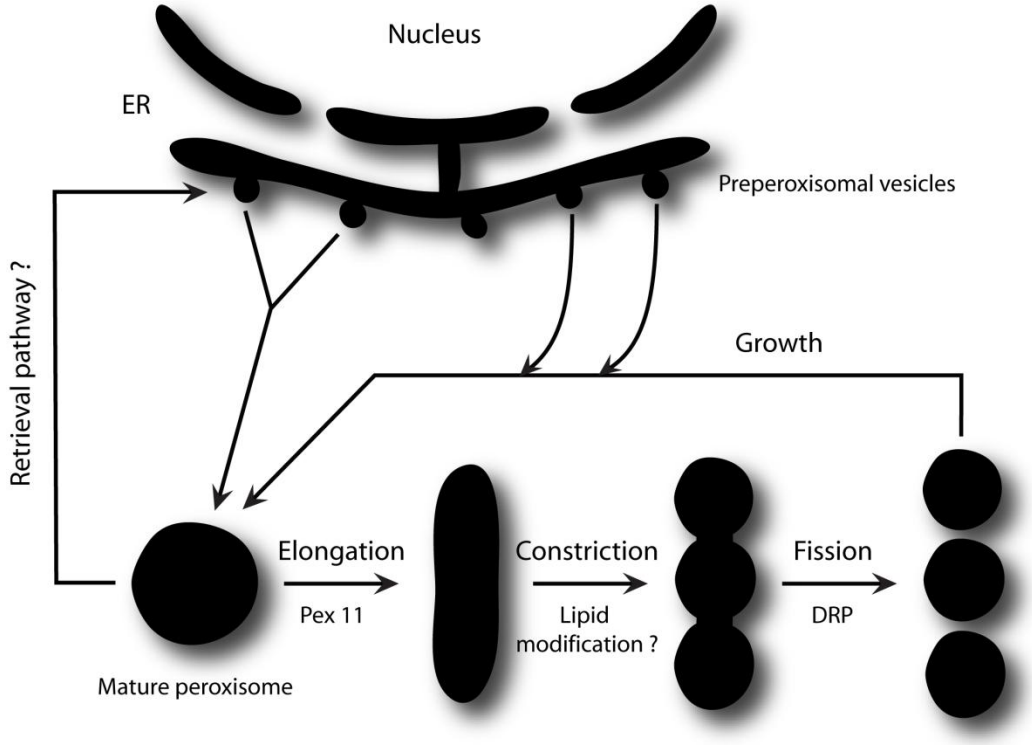
1.4 Peroxisome multiplication

1.4.1 *De novo* synthesis of peroxisomes from the ER

Cells lacking one of the biogenic peroxins Pex3p and Pex19p, along with Pex16p in mammals, are devoid of peroxisomal remnants. However, when the corresponding wild-type gene is reintroduced, mature peroxisomes are capable of forming *de novo* (Matsuzono *et al*., 1999; South *et al*., 2000; Kim *et al*., 2006) (Figure 1-1). This ability to form *de novo* suggested that peroxisomes were capable of arising from another organelle present within the cell. Morphometric and biochemical analysis suggested that this organelle is the ER.

Previous studies have illustrated a tight association between peroxisomes and specialized regions of the ER (Novikoff and Novikoff, 1972; Yamamoto and Fahimi, 1987; Grabenbauer *et al*., 2000). Three-dimensional reconstructions displayed membrane continuities between specialized regions of the ER and

Figure 1-1: A model of peroxisome biogenesis and division. Peroxisomal membrane proteins initially localize to the ER where the concentrate into discrete puncta. Budding of these specialized ER compartments gives rise to immature peroxisomes that mature either through the import of additional matrix and membrane proteins or the fusion with other immature preperoxisomal vesicles to give rise to mature, fully functional peroxisomes. Division of peroxisomes occurs through three distinct steps: peroxisome elongation, membrane constriction and membrane scission. Pex11 family proteins are thought to play the primary role in peroxisome elongation, while the final scission step is mediated by the dynamin-related proteins (DRPs). Membrane constriction is less understood but may require a modification of membrane lipid composition. Taken from Fagarasanu (2008).


Nucleus
ER
Preperoxisomal vesicles
Retrieval pathway ?
Growth
Elongation
Pex 11
Constriction
Lipid
modification ?
Fission
DRP
Mature peroxisome

peroxisomes, as well as enrichment of peroxisomal membrane proteins in constricted ER protrusions of mouse dendritic cells (Tabak *et al*., 2003). Additional evidence came from pulse-labeling experiments in the yeast *Yarrowia lipolytica* in which Pex2p and Pex16p were found to be *N*-linked glycosolated, a protein modification specific to the ER (Titorenko and Rachubinski, 1998). Phylogenetic studies of yeast and rat peroxisomal proteomes have suggested that most peroxins involved in biogenesis or maintenance are evolutionarily related to the ER (Gabaldon *et al*., 2006). Perhaps the most compelling support for an ER role in peroxisome biogenesis has come from time-lapse live cell imaging (Hoepfner *et al*., 2005; Tam *et al*., 2005). Upon reintroduction of the *PEX3* gene in *pex3Δ* cells, fluorescently labeled Pex3p first localized throughout the perinuclear ER and later concentrated at discrete foci before budding from the ER *en route* to forming the peroxisome (Hoepfner *et al*., 2005). It was later found that the membrane-spanning region of Pex3p encompassed by the first 46 amino acids was sufficient for the targeting of Pex3p to these discrete foci within the ER but was unable to fulfill the biogenic function of full-length Pex3p (Tam *et al*., 2005). Pex3p was also shown to be the membrane receptor for Pex19p, a peroxisomal membrane protein receptor and/or chaperone (Fang *et al*., 2004). This may suggest that the ER serves not only as the initiation site for *de novo* peroxisome formation but also as the site of some, if not all, peroxisomal membrane protein insertions.

While *de novo* peroxisome formation can occur, this process in *S. cerevisiae* was shown to be relatively inefficient compared with the process of peroxisome growth and division (Motley and Hettema, 2007). Barring a catastrophic loss of all peroxisomes in a cell, the ER's principal role in peroxisome biogenesis has been proposed to be the contribution of both membrane proteins and lipids to existing peroxisomes for use in their growth and division (Motley and Hettema, 2007).

1.4.2 Peroxisome division

In *S. cerevisiae*, the regulation of peroxisome abundance has traditionally been investigated using cells grown in fatty acid-containing medium to induce peroxisome proliferation. Under these conditions, peroxisomes become enlarged, and the activity of the peroxisome fission machinery is increased. The Pex11 protein family, consisting of Pex11p, Pex25p, and Pex27p, has been shown to have a major role in peroxisome proliferation (Erdmann and Blobel, 1995; Smith *et al.*, 2002; Rottensteiner *et al.*, 2003; Tam *et al.*, 2003). Cells lacking any of these proteins display fewer and enlarged peroxisomes, whereas their overproduction results in the presence of smaller and more numerous peroxisomes. Major caveats in using yeast grown in fatty acid-containing medium to investigate the regulation of the peroxisome population of a cell is that cells exhibit a drastic reduction in their rate of cellular division and peroxisome division is uncoupled from cell division. As a result, relatively little is known

about the mechanism of constitutive peroxisome division, which functions in actively growing cells with a normal cell cycle. It has previously been proposed that peroxisome division, whether linked to peroxisome proliferation or cell cycle-related duplication, would be dependent on the same set of divisional proteins (Yan *et al*., 2005) and would most likely consist of three major steps: peroxisome elongation, constriction and fission (Figure 1-1).

1.4.3 Peroxisome elongation

One of the first peroxins implicated in peroxisome division was Pex11p (Erdmann and Blobel, 1995; Marshall *et al*., 1995). In *S. cerevisiae*, cells deficient for Pex11p exhibit a decreased number of enlarged peroxisomes, while overproduction of Pex11p results in an increased number of peroxisomes in cells compared to the wild-type condition (Erdmann and Blobel, 1995). Cells overexpressing the *PEX11* gene often contain elongated peroxisomes, implicating Pex11p in the elongation step of peroxisome division (Marshall *et al*., 1995; Yan *et al*., 2005; Opalinski *et al*., 2011). Two additional proteins, Pex25p (Smith *et al*., 2002) and Pex27p (Rottensteiner *et al*., 2003; Tam *et al*., 2003), were later identified to have weak amino acid sequence similarity to Pex11p and found to play a role in maintaining the peroxisome population under conditions of peroxisome induction. Together, these three proteins form a family of proteins with low homology known as the Pex11 family of proteins involved in peroxisome division.

All three members of the Pex11p family are thought to be peripheral membrane proteins, although some controversy still remains in regards to Pex11p,. Single and double deletions of *PEX11* family members result in a reduced number of enlarged peroxisomes. Along with their roles in peroxisome division, Pex11p has been implicated in the efficient exchange of fatty acid metabolites across the peroxisomal membrane (van Roermund *et al*., 2000), while Pex25p has been shown to act in PTS1-mediated import of matrix proteins (Smith *et al*., 2002). Pex11 family proteins have previously been shown to self-interact and to interact with each other, suggesting that homo- and heterooligermization may play a role in regulating their function (Marshall *et al*., 1995; Li and Gould, 2003; Rottensteiner *et al*., 2003; Tam *et al*., 2003).

Structural analysis of Pex11 family proteins has shown that they exhibit extensive amino acid sequence similarity to the ligand-binding domain of nuclear hormone receptors (Barnett *et al*., 2000), suggesting a role for Pex11p in phospholipid binding and membrane modification. Recently, Pex11p has been implicated in both inducing and sensing membrane curvature (Opalinski *et al*., 2011; Koch and Brocard, 2011). It is yet to be determined whether these same membrane modifying properties are also responsible for Pex11p's role in metabolite transport across the peroxisomal membrane.

1.4.4 Constriction of the peroxisomal membrane

The molecular mechanisms underlying peroxisome constriction have remained largely unknown. Studies in *Y. lipolytica* have demonstrated an interesting mechanism by which membrane composition is modified in response to a signal from within the peroxisomal matrix. *Yl*Pex16p is a peripheral membrane protein present on the inner leaflet of the peroxisomal membrane which negatively regulates peroxisome division (Eitzen *et al*., 1997; Guo *et al*., 2003). *Yl*Pex16p regulates peroxisome division by inhibiting the formation of diacylglycerol, a potent inducer of membrane curvature. Accumulation of matrix proteins within the lumen of the peroxisome results in the accumulation of acyl-CoA oxidase on the peroxisomal inner membrane. This enzyme interacts with and suppresses *Yl*Pex16p-mediated inhibition of diacylglycerol formation (Guo *et al*., 2007). This interaction results in the formation of cone-shaped diacylglycerol in the outer leaflet of the peroxisomal membrane, causing membrane curvature and constriction. These sites of constriction are then acted upon by the cytosolic dynamin-related protein (DRP), *Yl*Vps1p, which executes the final scission of the peroxisomal membrane (Guo *et al*., 2007). The mechanism of peroxisome division initiation from a signal within the peroxisome is yet to be demonstrated for other organisms, and the proteins involved are likely to be different. In *S. cerevisiae*, a Pex16p homologue has not been identified, while mammalian Pex16p has been shown to play a role in peroxisome biogenesis from the ER rather than in peroxisome division (Honsho *et al*., 1998). More recently, Pex11p has been proposed to contain an amphipathic helix motif capable of inducing

membrane curvature both *in vitro* using small unilamellar vesicles and in the yeast *Hansenula polymorpha* cell itself (Opalinski *et al*., 2011), and may act as a substitute for membrane reorganization.

1.4.5 Final scission of peroxisomes

Once the peroxisomal membrane is constricted, its final scission is performed by DRPs. DRPs constitute a superfamily of large GTPases that function primarily in vesicle scission. Dynamins are thought to function as mechanochemical enzymes that use GTPase-dependent conformational changes to physically drive membrane fission (Praefcke and McMahon, 2004). While DRPs are capable of both membrane constriction and scission (Praefcke and McMahon, 2004; McMahon and Gallop, 2005), cells deficient for peroxisomal DRPs often display a single elongated, constricted peroxisome resembling "beads on a string" (Hoepfner *et al*., 2001; Koch *et al*., 2003). This observation suggests that peroxisomes in yeast are capable of membrane constriction, but not fission, in the absence of DRPs. Thus, membrane constriction and final scission are distinct steps in peroxisome division and require distinct molecular components (Schrader, 2006).

Initial studies identified Vps1p as a DRP involved in peroxisome division (Hoepfner *et al*., 2001). Cells lacking Vps1p show a drastic reduction in peroxisome numbers, with many cells containing only a single enlarged peroxisome. Vps1p was found to colocalize partially with peroxisomes and be

involved in peroxisome fission under both conditions of peroxisome induction and noninduction (Hoepfner *et al*., 2001; Li and Gould, 2003). While Vps1p appears to play the dominant role in peroxisome fission, a second DRP, Dnm1p, was found to affect peroxisome division. Dnm1p, which is normally involved in mediating mitochondrial fission, was also found to play a role in peroxisome fission, especially under conditions of peroxisome induction (Kuravi *et al*., 2006; Motley *et al*., 2008). Deletion of *DNM1* from cells already lacking Vps1p results in a further decrease in the peroxisome population, and nearly all cells contain a single peroxisome. Interestingly, these single enlarged peroxisomes elongate to form tubular structures that span from mother cell to bud. These structures are still correctly partitioned at cell division, with the final scission step occurring as a result of cytokinesis (Hoepfner *et al*., 2001; Kuravi *et al*., 2006; Fagarasanu *et al.*, 2009). While Fis1p has been shown to recruit Dnm1p to both the mitochondrial and peroxisomal membranes (Kuravi *et al*., 2006; Motley *et al*., 2008), the molecular components responsible for recruitment of Vps1p to the peroxisomal membrane remain to be determined.

1.5 Focus of this thesis

This thesis reports the characterization of a newly recognized peroxin, Pex34p, encoded by the open reading frame *YCL056c* of *S. cerevisiae*. Classical cell biological and biochemical techniques were combined with *in vivo* fluorescence confocal microscopy to elucidate the function of Pex34p in

peroxisome division. Pex34p was shown to be an integral membrane protein that positively regulates peroxisome division. Pex34p physically and genetically interacts with itself and with the three members of the Pex11 protein family: Pex11p, Pex25p and Pex27p. Using both inducible and constitutively expressed peroxisomal markers, we present data on the role of Pex34p in the division of peroxisomes under both conditions of peroxisome induction and noninduction. Using these techniques, we elucidated the similarities and differences of peroxisome proliferation versus constitutive division, identified secondary roles for Pex34p along with Pex25p in maintaining the mature peroxisome population and further our understanding of the order in which Pex34p, Pex11p, Pex25p and Pex27p function in the process of peroxisome division.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 List of chemicals and reagents

2-mercaptoethanol	BioShop
2-(*N*-Morpholino) ethanesulfonic acid (MES)	Sigma-Aldrich
2,4,6-tri-(dimethylaminomethyl) phenol (DMP-30)	Marivac
5-bromo-4-chloro-3-indolyl-β-D-galactoside	Rose Scientific
acetone	Fisher
acrylamide	Roche
agar	Difco
agarose, UltraPure	Invitrogen
albumin, bovine serum	Roche
ammonium chloride (NH_4Cl)	EM Science
ammonium persulfate (APS)	BDH
ammonium sulfate ($(NH_4)_2SO_4$)	BDH
ampicillin	Sigma-Aldrich
anhydrous ethyl alcohol (ethanol)	Commercial Alcohols
Brij 35	EM Science
bromophenol blue	BDH
chloroform	Fisher
complete protease inhibitor cocktail tablets	Roche
complete supplement mixture (CSM)	BIO 101

D-(+)-galactose	EM Science
D-(+)-glucose	EM Science
DDSA, specially distilled	Marivac
dimethyl sulfoxide (DMSO)	Caledon
dithiothreitol (DTT)	Fisher
ethidium bromide	Sigma-Aldrich
ethylenediaminetetraacetic acid (EDTA)	EM Science
glass beads	Sigma-Aldrich
glycerol	EM Science
glycine	Roche
isoamyl alcohol	Fisher
isopropanol (2-propanol)	Fisher
L-histidine	Sigma-Aldrich
lithium acetate	Sigma-Aldrich
L-leucine	Sigma-Aldrich
magnesium sulfate ($MgSO_4$)	Sigma-Aldrich
methanol	Fisher
methyl nadic anhydride (MNA)	Marivac
N,N,N',N'-tetramethylethylenediamine (TEMED)	EM Science
N, N'-dimethylformamide (DMF)	BDH
Nycodenz	BioLynx
oleic acid	Fisher
peptone	Difco

phenol, buffer saturated	Invitrogen
polyethylene glycol, M.W. 3350 (PEG)	Sigma-Aldrich
Ponceau S	Sigma-Aldrich
potassium chloride (KCl)	BDH
potassium permanganate ($KMnO_4$)	BDH
potassium phosphate, dibasic (K_2HPO_4)	EM Science
potassium phosphate, monobasic (KH_2PO_4)	EM Science
propylene oxide	Marivac
salmon sperm DNA, sonicated	Sigma-Aldrich
skim milk, powdered	Carnation
sodium acetate	EM Science
sodium carbonate (Na_2CO_3)	BDH
sodium chloride (NaCl)	EM Science
sodium dodecylsulfate (SDS)	Bio-Rad
sodium hydroxide	Sigma-Aldrich
sodium periodate ($NaIO_4$)	Sigma-Aldrich
sorbitol	EM Science
sucrose	EM Science
TAAB 812 resin	Marivac
trichloroacetic acid	EM Science
tris(hydroxymethyl)aminomethane (Tris)	Roche
Triton X-100	VWR
tryptone	Difco

Tween 20	Sigma-Aldrich
Tween 40	Sigma-Aldrich
uracil	Sigma-Aldrich
xylene cyanol FF	Sigma-Aldrich
yeast extract	Difco
yeast nitrogen base without amino acids (YNB)	Difco

2.1.2 List of enzymes

Easy-A cloning enzyme	Stratagene
restriction endonucleases	NEB
T4 DNA ligase	NEB
Zymolyase 100T	ICN

2.1.3 Molecular size standards

1kb DNA ladder (500-10,000 bp)	NEB
prestained protein marker, broad range (6-175 kDa)	NEB

2.1.4 Multicomponent systems

BigDye Terminator Cycle Sequencing Ready Reaction Kit	Applied Biosystems
QIAprep Spin Miniprep Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR purification Kit	Qiagen
Ready-To-Go PCR Beads	Biosciences
SuperSignal west femto maximum sensitivity substrate	Thermo Scientific

2.1.5 Plasmids

pGREG576	Jansen *et al.*, 2005
pGAD424	Clontech
pGBT9	Clontech
pBY011	Bhullar, Harvard Institute of Proteomic

2.1.6 Antibodies

The antibodies used in this study are listed in Tables 2-1 and 2-2.

Table 2-1 Primary Antibodies

Specificity	Type		Dilution	Source
Anti-GFP	rabbit		1:5000	Eitzen *et al*., 1996
Y. lipolytica thiolase	guinea pig	N-3	1:10,000	Eitzen *et al*., 1996
S. cerevisiae Pex3p	rabbit	P84-final	1:4000	Fagarasanu *et al*., 2006
S. cerevisiae Pex27p	guinea pig	Q10-final	1:4000	Fagarasanu *et al*., 2006
S. cerevisiae Sdh2p	rabbit		1:5000	Dibrov *et al*., 1998

Table 2-2 Secondary Antibodies

Specificity	Type	Dilution	Source
Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG	donkey	1:20,000	Amersham Bioscience
HRP-conjugated anti-guinea pig IgG	goat	1:20,000	Sigma-Aldrich

2.1.7 Oligonucleotides

The oligonucleotides used in this study were synthesized by Sigma-Genosys (Oakville, Ontario) and are listed in Table 2-3.

Table 2-3 Oligonucleotides

Name	Sequence	Application
0021-PEX25, PrA	GTAAATATTTTGGTGAATTTGCTCG	*PEX25* Checking Primer
0022-PEX25, PrD	TTCTTGGTTTGGCAGAATAAGTTAC	*PEX25* Checking Primer

0023-YOR193w, PrA	TTATCAACAGATTCACATTCCAAGA	*PEX27* Checking Primer
0024-YOR193w, PrD	ATGATAAAGTCACAAGAACAAAGGC	*PEX27* Checking Primer
0075-PEX11f	ATTGGATCCAGCTCACTAACGACATTGGC CAGT	*PEX11*Checking Primer
0076-PEX11r	ATTGGATCCTAACTATTCCTTCCTAATCCT CT	*PEX11* Checking Primer
0545-prDyjl185c	TCATGTTAATTATCTGGAGAGCACA	*YJL185c* Checking Primer
0650-MF-prC/YJL185c	TAAAGAAACTTACAAATGCCCAAAG	*YJL185c* Checking Primer
0775-RP-GFP +200bp5'out	CGGGAAAAGCATTGAACACCA	GFP Checking Primer
0984-AF-INP2D	GCACTTGATCTTTTCTCAAGACTTC	*INP2* Checking Primer
1008-BK-BK184	GGGTTGTTAGTATGTGTATCG	*POT1* Checking Primer
1316-DW-YIL160c-Pot1p, 3-ketoacyl-CoA thiolase-3WebA	GGGGTTGTTAGTATGTGTATCGGTACTG GTATGGGTGCCGCCGCCATCTTTATTAA AGAAGGCGGTGGCGGTGAAGCTCAAAA ACTTAAT	*POT1-GFP*
1317-DW-YIL160c-Pot1p 3-ketoacyl-CoA thiolase-3WebB	AAATATTGAAAATGGAAAATTATAAACAA ATTGATAAACTACGTAATAGCTTTTACAAA GACGGTATCGATAAGCTT	*POT1-GFP*
1344-DW-YDR329c-Pex3p-3WebA	GATCTGAGCGCCAGCGTATACAGCAACTT TGGCGTCTCCAGCTCGTTTTCCTTCAAGC CTGGCGGTGGCGGTGAAGCTCAAAAACT TAAT	*PEX3-mRFP*
1345-DW-YDR329c-Pex3p-3WebB	TCAATATATCAACCTATTTCTTCCCTTTCTC TTCTTTTCTCCAAGACGCCCGTTAAATCGA CGGTATCGATAAGCTT	*PEX3-mRFP*
1678-FM5	TCCATAGTGTTAGAGTCTCTAAT	*PEX3* Checking Primer
1679-FM6	TCACGCGCTCCCACTTGAA	mRFP Checking Primer
1775-MF-Inp1koF	AAGGTCTACATTTTTCGTCTGATAACTCT CAGGAAATTAAACAAAGTGGTAGATTGT ACTGAGAGTGCAC	*INP1* Deletion

1776-MF-Inp1koR	ATTTATATTCACATTGTATACTCCTTCACTT TGGTTTACACCTACATTCACTGTGCGGTA TTTCACACCG	*INP1* Deletion
2071-NN-checkINPP	ATTCCTTCACCTATGAAGGATA	*INP1* Checking Primer
2198-BK638	GAAAAACCGTTGTCTCCATCTACTACTTC AAAGACTTCATCAAGTAATAGTATAATCA ATAGATTGTACTGAGAGTGCAC	*PEX11* Deletion
2199-BK639	AAGGGTCGAATCAAACATAAGCGGAGAAT AGCCAAATAAAAAAAAAAAGATGAAAAGA AAGCTGTGCGGTATTTCACACCG	*PEX11* Deletion
2200-MF-Vps1-del-F	CTTTTATAGCACCAAAATAAGGACCGTACG AAAACTGCACATTTTATATTATCAGATATCA GATTGTACTGAGAGTGCAC	*VPS1* Deletion
2201-MF-Vps1-del-R	AAATATTAGGGAGAAATACTCAAAACCAA GCTTGAGTCGACCGGTATAGATGAGGAAA ACCTGTGCGGTATTTCACACCG	*VPS1* Deletion
2573-FM119-INP2KO-fw	CCCTCAATAACTGCTCGGCATACCTCACG CTTATTGCAACAAGTTTGTTTTTACTTACTT AGATTGTACTGAGAGTGCAC	*INP2* Deletion
2574-FM120-INP2KO-rev	TAGAAAATATGATTAAAGTGTAATTAGTTAT TTCAAAGTACATATTAAAATATATTATCACT GTGCGGTATTTCACACCG	*INP2* Deletion
2888-MF-YjlKOF	TGTATTCAGGGCTTAAAATACTAAAATTTG GTGGTCAGTACAGCTCATTAAGATTGTAC TGAGAGTGCAC	*YJL185c* Deletion
2889-MF-YjlKOR	CTACCATTCTCTGTCCAAAAACCTAGAAA GTAAAGTCCAGCCAAACCAACTGTGCGG TATTTCACACCG	*YJL185c Deletion*
3293-YCL056c KO rev	TCTGAGGTAAGAAGTAAAGAGGAGATGA ATAAAAGTAAAGGAAGAAGAAAAGAAAG TTGACTGTGCGGTATTTCACACCG	*PEX34* Deletion
3294-YCL056c KO fwd	TCTAAGGATGCTTTCTTGAAGGAGCTGGG CTTACAGAACTAAATTCGTTCAAGCATAAA AAGATTGTACTGAGAGTGCAC	*PEX34* Deletion
3321-RT7-FWD-BamHI-Ycl056c	ATTGGATCCAAAAGTCGCACTTGAAAAGT TG	*PEX34* Checking Primer, YEp13-*PEX34*
3322-RT8-REV-Ycl056c-BamHI	ATTGGATCCTCTAATGGTGGTACGTTATCC	*PEX34* Checking Primer, YEp13-*PEX34*

3669-RT25-Fis1 KO-FWD	TACCTTGCGTAAAAACGGCACATAGAAGC ACAGATCAGAGCACAGCCATACAACATAA GTAGATTGTACTGAGAGTGCAC	*FIS1* Deletion
3670-RT26-FIS1 KO-REV	TACGCGTGCGTGCGATTCATTCTTATGTAT GTACGTATGTGCTGATTTTTTATGTGCTTG CTGTGCGGTATTTCACACCG	*FIS1* Deletion
3673-RT27-BamHI-Fis1-FWD	ATTGGATCCCAGTTCAAATAACATGTGTCC AT	*FIS1* Checking Primer
3674-RT28-Fis1-BamHI-REV	ATTGGATCCCCCTTTGAGCAGCGCCTA	*FIS1* Checking Primer
3686-RT29-Rec1-Ycl056c	GAATTCGATATCAAGCTTATCGATACCGTC GACAATGGTTTCGAAGAAAAATACGGCT	pGREG576-*PEX34*
3687-RT30-Rec2-Ycl056c	GCGTGACATAACTAATTACATGACTCGAGG TCGACTTATACAATTATTCTACAAAGTGTT	pGREG576-*PEX34*
3798-MDH2 3WebA	CAGTTAAAGAAAAATATCGATAAGGGCTTG GAATTCGTTGCATCGAGATCTGCATCATCTG GTGAAGCTCAAAAACTTAAT	*MDH2-GFP*
3799-MDH2 3WebB	GACTGGCTTAACGGGAATATTATCAATTTG CTGCATTCTTATGCTTCGGTCCGATGCTCA GCTGACGGTATCGATAAGCTT	*MDH2-GFP*
3800-MDH2-CheckingC	GTCCAATTTGTTTCTTTGTTATTGG	*MDH2* Checking Primer
3893-RT35-PrB Ycl056c	TGACCACGCCTAGGTTTTC	*PEX34* Checking Primer
3894-RT36-PrC Ycl056c	ATATTACTGCTGCTTTACTCC	*PEX34* Checking Primer
3897-RT37-F-Pex25 KO	TAGAGTCGATATCCTCAACTTCCCTGTAAG TCTTCACCTATAGAAACTGGTCGTAAAACA AGATTGTACTGAGAGTGCAC	*PEX25* Deletion
3898-RT38-R-Pex25 KO	GTAAAACATTATTCGCCACATATATATGTAC ATATCTATATGTATACATATTTTTATATACTG TGCGGTATTTCACACCG	*PEX25* Deletion
3899-RT39-F-Pex27 KO	GGATGATAGGATTTGAAGGTAGACTATGAC CTTTGTGTTAACTTGGACAATCGTTTTATCA GATTGTACTGAGAGTGCAC	*PEX27* Deletion
3900-RT40-R-Pex27 KO	TATAATATCAAACTAAAAAAACGAAATAA AGAGGGATGCAACGAACTTGGTCATCTGTT GCTGTGCGGTATTTCACACCG	*PEX27* Deletion

4068-Eco-Fis1-fwd	CCCGGGAATTCACCAAAAGTAGATTTTTGGCCAACTCT	pGAD424-*FIS1*, pGBT9-*FIS1*
4069-Eco-Fis1-rev	CCCGGGAATTCTTACCTTCTCTTGTTTCTTAAGAAGAAA	pGAD424-*FIS1*, pGBT9-*FIS1*
4070-Eco-Vps1-fwd	CCCGGGAATTCGATGAGCATTTAATTTCTACTATTAACAA	pGAD424-*VPS1*, pGBT9-*VPS1*
4071-Pst-Vps1-rev	CCCGCTGCAGCTAAACAGAGGAGACGATTTGACTAG	pGAD424-*VPS1*, pGBT9-*VPS1*
4072-Eco-Ycl056c-fwd	CCCGGGAATTCGTTTCGAAGAAAAATACGGCTGAAATC	pGAD424-*PEX34*, pGBT9-*PEX34*
4073-Bam-Ycl056c-rev GTGTTATTATG	CCCGGGATCCTTATACAATTATTCTACAAA	pGAD424-*PEX34*, pGBT9-*PEX34*
4105-Bam-Fis1-rev	CCCGGGATCCTTACCTTCTCTTGTTTCTTAAGAAGAAA	pGAD424- *FIS1*, pGBT9-*FIS1*
4106-Pex25-fwd	GAAGAGACAATTAGAATTGGAAGTGC	*PEX25* sequencing
4107-Pex11-fwd	ATGAGCATGAGGATCACAAGAAGG	*PEX11* sequencing
4108-Vps1-fwd	AAACTGACAAAGTGACAGGTGCC	*VPS1* sequencing
4109-Vps1-fwd2	GGTTATATCCCAGTTATCAATAGAGG	*VPS1* sequencing
4110-Vps1-fwd3	TGGTGCTAGAATTTCTTACGTATTCC	*VPS1* sequencing
4111-Vps1-fwd4	TGAACAAACTTACATCAATACAGCCC	*VPS1* sequencing
4112-Vps1-fwd5	TTCTCTATTGTCAAAAGAACCATTGCC	*VPS1* sequencing
4142-DW-Pex25-seq	CAATCACGGTAATTAAAGTACTTCTC	*PEX25* sequencing

2.1.8 Standard buffers and solutions

The compositions of routinely used buffered solutions are given in Table 2-4.

Table 2-4 Common Solutions

Solution	Composition	Reference
1x TBST	20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20	Huynh *et al*., 1988
1x Transfer Buffer	20 mM Tris-HCl, 150 mM glycine, 20% (v/v) methanol	Towbin *et al*., 1979; Burnette, 1981
5x SDS-PAGE running buffer	0.25 M Tris-HCl, pH 8.8, 2 M glycine, 0.5% SDS	Ausubel *et al*., 1989
10x TBE	0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA	Maniatis *et al*., 1982
2x sample buffer	20% (v/v) glycerol, 167 mM Tris-HCl, pH 6.8, 2% SDS, 0.005% bromophenol blue	Ausubel *et al*., 1989
6x DNA loading dye	0.25% bromophenol blue, 0.25% xylene cyanol, 30% (v/v) glycerol	Maniatis *et al*., 1982
Yeast Breakage Buffer	2% (v/v) Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0	Ausubel *et al*., 1989
Ponceau stain	0.1% Ponceau S, 1% TCA	Szilard, 2000
TE	10 mM Tris-HCl, pH 7.0-8.0 (as needed), 1 mM EDTA	Maniatis *et al*., 1982
LiAc/PEG	40% PEG, 0.1 M LiAc, 2.5 mM EDTA	Tam *et al*., 2005

2.2 Microorganisms and culture conditions

2.2.1 Bacterial strain and culture conditions

The *Escherichia coli* strains and culture medium used in this study are described in Tables 2-5 and 2-6, respectively. Bacteria were grown at 37°C. Cultures of 5 ml or less were grown in culture tubes in a rotary shaker at 200 rpm. Cultures greater than 5 ml were grown in flasks in a rotary shaker at 200 rpm. Culture volumes were approximately 20% of flask volume.

Table 2-5 *E. coli* Strains

Strain	Genotype	Source
DH5α	F^-, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F), U169, *rec*A1, *end*A1, *hsd*R17(r_k^-, m_k^+), *pho*A, *sup*E44, λ^-, *thi*-1, *gyr*A96, *rel*A1	Invitrogen

Table 2-6 Bacterial Culture Medium

Medium	Composition	Reference
LB[a,b]	1% tryptone, 0.5% yeast extract, 1% NaCl	Maniatis *et al*., 1982

[a] Ampicilin was added to 100 μg/ml for plasmid selection when necessary.
[b] For solid medium, agar was added to 1.5%.

2.2.2 Yeast strains and culture conditions

The *Saccharomyces cerevisiae* strains and culture media used in this study are listed in Tables 2-7 and 2-8, respectively. Yeast were grown at 30°C unless otherwise stated. Cultures of 10 ml or less were grown in 16 x 150 mm glass tubes in a rotating wheel. Cultures greater than 10 ml were grown in flasks

in a rotary shaker at 200 rpm. Culture volumes were approximately 20% of flask volumes. For overexpression, cultures were grown for 16 h in SCIM without uracil, at which time glucose and galactose were added to 0.2% and 1%, respectively. Images were acquired 90 min after addition of galactose.

Table 2-7 *S. cerevisiae* Strains

Strain	Genotype	Reference
BY4742	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	Giaver *et al.*, 2002
SFY526	*MATα, ura3-52, his3-200, ade2-101, lys2-80, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS::GAL1$_{UAS}$-GAL1$_{TATA}$-lacz, MEL1*	Harper *et al.*, 1993
POT1-mRFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-mRFP (NAT)*	This study
POT1-mRFP/ pGREG576-*PEX34*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-mRFP (NAT),* pGREG576-*PEX34 (URA3)*	This study
POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT)*	This study
POT1-GFP/pBY011	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT),* pBY011 *(URA3)*	This study
POT1-GFP/pBY011-*PEX34*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT),* pBY011-*PEX34 (URA3)*	This study
pex34Δ	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pex34:: LEU2*	This study
pex34Δ/POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2*	This study
pex34Δ/POT1-GFP/ pBY011	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2,* pBY011 *(URA3)*	This study
pex34Δ/POT1-GFP/ pBY011-*PEX34*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2,* pBY011-*PEX34 (URA3)*	This study

pex34Δ/POT1-GFP/ pBY011-*PEX11*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2,* pBY011-*PEX11 (URA3)*	This study
pex34Δ/POT1-GFP/ pBY011-*PEX25*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2,* pBY011-*PEX25 (URA3)*	This study
pex34Δ/POT1-GFP/ pBY011-*PEX27*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2,* pBY011-*PEX27 (URA3)*	This study
pex11Δ/POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex11::HIS3*	This study
pex11Δ/POT1-GFP/ pBY011	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex11::HIS3,* pBY011 *(URA3)*	This study
pex11Δ/POT1-GFP/ pBY011-*PEX34*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex11::HIS3,* pBY011-*PEX34 (URA3)*	This study
pex25Δ/POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex25::URA3*	This study
pex25Δ/POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex25::HIS3*	This study
pex25Δ/POT1-GFP/ pBY011	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex25::HIS3,* pBY011 *(URA3)*	This study
pex25Δ/POT1-GFP/ pBY011-*PEX34*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex25::HIS3,* pBY011-*PEX34 (URA3)*	This study
pex27Δ/POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex27::HIS3*	This study
pex27Δ/POT1-GFP/ pBY011	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex27::HIS3,* pBY011 *(URA3)*	This study
pex27Δ/POT1-GFP/ pBY011-*PEX34*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex27::HIS3,* pBY011-*PEX34 (URA3)*	This study
pex34Δ/pex11Δ/ POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2, pex11::HIS3*	This study
pex34Δ/pex25Δ/ POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2, pex25::URA3*	This study
pex34Δ/pex27Δ/ POT1-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2, pex27::HIS3*	This study

MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT)*	This study
pex34Δ/MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex34::LEU2*	This study
pex11Δ/MDH2-GFP	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex11::HIS3*	This study
pex25Δ/MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex25::URA3*	This study
pex27Δ/MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex27::HIS3*	This study
pex34Δ/pex11Δ/ MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex34::LEU2, pex11::HIS3*	This study
pex34Δ/pex25Δ/ MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex34::LEU2, pex25::URA3*	This study
pex34Δ/pex27Δ/ MDH2-GFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex34::LEU2, pex27::HIS3*	This study
pex25Δ/MDH2-GFP/ PEX3-mRFP	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex3::PEX3-mRFP (HYG), pex25::URA3*	This study
pex34Δ/pex25Δ/ MDH2-GFP/PEX3-mRFP	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), pex3::PEX3-mRFP (HYG), pex34::LEU2, pex25::URA3*	This study
yjl185cΔ/POT1-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), *yjl185c::HIS3*	This study
fis1Δ/POT1-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), *fis1::URA3*	This study
pex34Δ/yjl185cΔ/ POT1-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), pex34::LEU2, *yjl185c::HIS3*	This study
yjl185cΔ/fis1Δ/POT1-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), *fis1*::*URA3*, *yjl185c::HIS3*	This study
pex34Δ/fis1Δ/POT1-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), *fis1*::*URA3*, *pex34::LEU2*	This study
pex34Δ/fis1Δ/ yjl185cΔ/POT1-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1:: POT1-GFP (NAT), *fis1*::*URA3*, *pex34::LEU2*, *yjl185c::HIS3*	This study
inp2Δ/MDH2-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), *inp2::LEU2*	This study

pex27Δ/inp2Δ/MDH2-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), *inp2*::LEU2, *pex27::HIS3*	This study
vps1Δ/MDH2-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), *vps1::URA3*	This study
vps1Δ/inp2Δ/MDH2-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, mdh2:: MDH2-GFP (NAT), *vps1*::URA3, *inp2::LEU2*	This study

Table 2-8 Yeast Culture Medium

Medium	Composition	Reference
Nonfluorescent medium	6.61 mM KH_2PO_4, 1.32 mM K_2HPO_4, 4.06 mM $MgSO_4 \cdot 7H_20$, 26.64 mM $(NH_4)SO_4$, 1 × CSM, 2% glucose, 1% agarose	Tam *et al*., 2005
SCIM	0.67% YNB, 0.5% yeast extract, 0.5% peptone, 0.5% (w/v) Tween 40, 0.3% glucose, 0.3% (v/v) oleic acid, 1 × CSM	Fagarasanu *et al*., 2006
SM	0.67% YNB, 2% glucose, 1× CSM without leucine, uracil, or histidine as required	Tam *et al*., 2005
YEPD	1% yeast extract, 2% peptone, 2% glucose	Rose *et al*., 1988
YPBO	0.3% yeast extract, 0.5% peptone, 0.5% K_2HPO_4, 0.5% KH_2PO_4, 0.2% (w/v) Tween 40 or 1% (v/v) Brij 35, 1% (v/v) oleic acid	Kamiryo *et al*., 1982

2.3 Introduction of DNA into microorganisms

2.3.1 Chemical transformation of *E. coli*

Plasmid DNA was introduced into Subcloning Efficiency, chemically competent *E. coli* DH5α cells, as recommended by the manufacturer (Invitrogen). Essentially, 1 to 2 µl of ligation reaction (Section 2.5.7) or 0.5 µl (0.25 µg) of plasmid DNA was added to 25 µl of cells. The mixture was incubated on ice for 30 min, subjected to a 20 sec heat shock at 37ºC, and chilled on ice for

2 min. 975 µl of LB medium (Table 2-6) were added, and the cells were incubated in a rotary shaker for 60 min at 37ºC. Cells were spread onto LB agar plates (Table 2-6) containing ampicillin and incubated overnight at 37ºC.

2.3.2 Chemical transformation of yeast

5 ml yeast cultures were grown to an OD_{600} of ~0.8 in YEPD medium. Cells were pelleted for 3 min at 2000 x *g*, then rinsed in 500 µl of 0.1 M LiAc. Cells were mixed with 10 µl of 10 mg sheared and freshly boiled salmon sperm/ml, and 0.5 µl of plasmid DNA from a standard mini prep or 25 µl of PCR reaction with ~50 bp homology for chromosomal integration. Cells were then resuspended in 500 µl of LiAc/PEG mixture (Table 2-4). Cells were mixed by inversion with 53 µl of DMSO and incubated for 15 min at room temp, then heat shocked for 15 min at 42°C. Cells were harvested at 950 x *g* for 3 min, rinsed once in 1 ml of sterile water, resupended in 100 µl of sterile water and plated onto selective medium.

2.4 Isolation of DNA from microorganisms

2.4.1 Isolation of plasmid DNA from bacteria

Single bacterial colonies were inoculated into 2 ml of LB medium containing ampicillin and incubated for 8 h at 37ºC. Cells were harvested by microcentrifugation, and plasmid DNA was isolated using a QIAprep Spin Miniprep Kit according to the manufacturer's instructions (Qiagen). This method is based on the alkaline lysis of bacterial cells, followed by adsorption of DNA

onto silica in the presence of high salt and elution of DNA in low salt buffer. Plasmid DNA was usually eluted in 50 µl of the supplied elution buffer.

2.4.2 Isolation of chromosomal DNA from yeast

Yeast genomic DNA was prepared as recommended by Ausubel *et al*. (1989). Cells were grown overnight in 5 ml of YEPD medium, harvested by centrifugation for 5 min at 2,000 × *g*, washed twice in 10 ml of sterile water, and transferred to a 1.5 ml microcentrifuge tube. 400 µl of breakage buffer (Table 2-4) and 100 µl of glass beads were added to cells, which were vortexed for 3 min. 250 µl of phenol and 250 µl of chloroform/isoamyl alcohol (24:1) were added to the cells, which were again vortexed for 3 min. The organic and aqueous phases were separated by centrifugation at 16,000 × *g* for 10 min at room temperature. The aqueous phase was transferred to a fresh tube, and DNA was pelleted by the addition of 1 ml of absolute ethanol and 100 µl of 20% sodium acetate, incubation at -20°C for 20 to 60 min and centrifugation at 16,000 × *g* for 5 min at 4°C. The pellet was washed once with 1 ml of 70% (v/v) ethanol, dried in a rotary vacuum desiccator and dissolved in 50 µl of TE.

2.5 DNA manipulation and analysis

Unless otherwise indicated, reactions were carried out in 1.5-ml microcentrifuge tubes, and microcentrifugation was performed in an Eppendorf microcentrifuge at 16,000 × *g*.

2.5.1 Amplification of DNA by the polymerase chain reaction (PCR)

PCR was used to amplify specific DNA sequences or to introduce modifications in the amplified DNA sequence. Primer design, reaction components and cycling conditions were performed following standard protocols (Innis and Gelfand, 1990; Saiki, 1990). A reaction usually contained 0.5 µl of yeast genomic DNA or 0.2 µl of plasmid DNA, 25 pmol of each primer, 0.25 mM of each dNTP, and 1 U of Easy-A high-fidelity polymerase in 5 µl of the supplied reaction buffer (Stratagene) at a final volume of 50 µl. Reactions were performed in 0.2-ml microcentrifuge tubes in a Model 2720 thermocycler with a hot top attachment (Applied Biosystems). Alternatively, Ready-to-Go PCR Beads were used as recommended by the manufacturer (Amersham Biosciences).

2.5.2 Digestion of DNA by restriction endonucleases

In general, 1 to 2 µg of plasmid DNA or purified DNA was digested by restriction endonucleases for 1 to 1.5 h according to the manufacturer's instructions. Digestion was immediately terminated by agarose gel electrophoresis of the DNA fragments.

2.5.3 Separation of DNA fragments by agarose gel electrophoresis

DNA fragments in solution were mixed with 0.2 volume of 6 × DNA loading dye (Table 2-4) and separated by electrophoresis in 0.8% agarose gels in 1 × TE (Table 2-4) containing 0.5 µg of ethidium bromide/ml. Gels were subjected to electrophoresis at 10 V/cm in 1 × TE, and DNA fragments were

subsequently visualized on an ultraviolet transilluminator (Photodyne, Model 3-3006).

2.5.4 Purification of DNA fragments from agarose gel

A DNA fragment of interest was excised from the agarose gel using a razor blade. DNA was extracted from the agarose slice using the QIAquick Gel Extraction Kit according to manufacturer's instructions (Qiagen). This method is based on the dissolution of agarose gel and adsorption of DNA to a silica membrane in the presence of a high concentration of chaotropic salts, followed by washing and elution of DNA in the presence of low salt. DNA was usually eluted in 30 to 50 µl of the supplied elution buffer.

2.5.5 Purification of DNA from solution

Contaminants (small oligonucleotides, salts, enzymes, etc.) were removed from a DNA solution using the QIAquick PCR Purification Kit as described by the manufacturer (Qiagen). The principle of this method is similar to that of the QIAquick Gel Extraction Kit (Section 2.5.4), except that no dissolution of agarose gel was involved. DNA was usually eluted in 30 to 50 µl of the supplied elution buffer.

2.5.6 Ligation of DNA fragments

DNA fragments treated with restriction endonucleases and purified as described in Section 2.5.4 were ligated using 1 µl of T4 DNA ligase in the

buffer supplied by the manufacturer (NEB). The reaction was typically conducted in a volume of 10 µl, with the molar ratio of plasmid to insert being 1:3, and incubated overnight at 16ºC.

2.5.7 DNA sequencing

DNA sequencing was performed using the BigDye Terminator v1.1/3.1 Cycle Sequencing Ready Reaction Kit as described by the manufacturer (Applied Biosystems). This method is based on the method of Sanger *et al*. (1977) and involves the random incorporation of fluorescent dideoxy terminators during the elongation of DNA sequences with a modified version of *Taq* DNA polymerase. Essentially, a reaction contained 1 µl of plasmid DNA, 3.2 pmol of primer, 3 µl of Terminator Ready Reaction Mix, and 2.5 µl of the supplied 5x buffer in a total volume of 20 µl. The reaction was subjected to cycle sequencing using the Model 2720 thermocycler with hot top attachment and the following conditions: 1 cycle at 96ºC for 2 min; 25 cycles at 96ºC for 46 sec, 50ºC for 51 sec and 60ºC for 4 min 10 sec; 1 cycle at 4ºC to hold until ready to purify. Reaction products were precipitated by the addition of 80 µl of 75% (v/v) isopropanol for 20 min at room temperature, subjected to microcentrifugation at 16,000 × *g* for 20 min, washed twice with 250 µl of 75% isopropanol, dried in a rotary vacuum dessicator and dissolved in 15 µl of Template Suppression Reagent. They were then heated at 95ºC for 2 min and immediately cooled on ice. Finally, they were separated by capillary electrophoresis, and fluorescence was detected and recorded by an ABI 310 Genetic Analyzer (Applied Biosystems).

2.6 Protein manipulation

2.6.1 Precipitation of proteins

Proteins were precipitated from solution by addition of TCA to a final concentration of 10% and incubation on ice for 30 min to overnight. Precipitates were collected by microcentrifugation at 16,000 × *g* for 30 min at 4ºC. Pellets were washed twice with 1 ml of ice-cold acetone, dried in a rotary vacuum dessicator and dissolved in 2 × sample buffer (Table 2-4).

2.6.2 Separation of proteins by electrophoresis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Ausubel *et al*. (1989). Protein samples were mixed with an equal volume of 2 × sample buffer containing 10 mM DTT, denatured by boiling for 5 min, and separated by electrophoresis on discontinuous slab gels. Stacking gels contained 3% acrylamide (30:0.8 acylamide:*N*,*N'*-methylene-bis-acrylamide), 60 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.1% (v/v) TEMED, and 0.1% ammonium persulfate. Resolving gels contained 10% acrylamide (30:0.8 acylamide:*N*,*N'*-methylene-bis-acrylamide), 370 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% (v/v) TEMED, and 0.043% ammonium persulfate. Electrophoresis was conducted in 1x SDS-PAGE running buffer (Table 2-4) at 50-200 V using a Bio-Rad Mini Protean II vertical gel system.

2.6.3 Detection of proteins by immunoblotting

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Bio-Rad) in 1x transfer buffer (Table 2-4) at 100 V for 1 h at 4°C using a Trans-Blot tank transfer system with plate electrodes (Bio-Rad). Proteins transferred to nitrocellulose were visualized by staining in Ponceau stain (Table 2-4) for several min and destaining in water. The nitrocellulose was incubated in blocking solution (5% skim milk powder, 1x TBST (Table 2-4)) with gentle agitation to prevent nonspecific binding of antibodies. Specific proteins on nitrocellulose were detected by incubation with primary antibody in blocking solution for 1 h at room temperature with gentle agitation. The membrane was then rinsed in blocking solution twice for 5 min each, in 1x TBST for 5 min, and again in blocking solution for 5 min. The nitrocellulose was then incubated with the appropriate HRP-labeled secondary antibody in blocking solution for 45 min. The membrane was then rinsed in blocking buffer twice for 5 min each, followed by three 5-min washes in 1x TBST. Antigen-antibody complexes were detected using an ECL Western Blotting Detection Kit according to the manufacturer's instructions (Amersham Biosciences) and exposure of the nitrocellulose membrane to X-Omat BT film (Kodak).

Used nitrocellulose could be reblotted using a Re-Blot Western Blot Recycling Kit according to the manufacturer's instructions (Chemicon). The nitrocellulose was incubated with 1x Antibody Stripping Solution at room temperature for 10 min with gentle agitation, rinsed with 1x TBST, and blotted as described above.

2.7 Subcellular fractionation of *S. cerevisiae* cells

2.7.1 Peroxisome isolation from *S. cerevisiae*

Cells grown in SCIM were harvested by centrifugation for 5 min at 6000 × *g* in a Beckman JA10 rotor at room temperature and washed twice with water. Cells were resuspended in 100 mM Tris-HCl, pH 9.4, at a concentration of 10 ml per g of wet cells and incubated at 30ºC for 30 min at 70 rpm to loosen the outer mannoprotein layer. Cells were collected by centrifugation at 2000 × *g* in a Beckman JS13.1 rotor for 8 min at 4ºC and washed once with Zymolyase buffer (50 mM potassium phosphate, pH 7.5, 1.2 M sorbitol, 1 mM EDTA). Cells were resuspended in Zymolyase buffer containing 1 mg of Zymolyase 100T/ml at a concentration of 8 ml per g of wet cells and incubated at 30ºC for 30 to 90 min with gentle agitation to convert them to spheroplasts. Spheroplasts were harvested by centrifugation at 1850 × *g* in a Beckman JS13.1 rotor for 8 min at 4ºC and washed once with buffer H (0.6 M sorbitol, 2.5 mM MES, pH 6.0, 1 mM EDTA, 1 × complete protease inhibitor cocktail (Roche)). Spheroplasts were then resuspended in 10 ml of buffer H, transferred to a homogenization mortar and disrupted by 15 strokes of a Teflon pestle driven by a stirrer motor (Model 4376-00, Cole-Parmer) at 34% of maximal speed. Cell debris, unbroken cells and nuclei were pelleted by centrifugation at 1,000 × *g* in a JS13.1 rotor for 7 min at 4ºC. The postnuclear supernatant (PNS) fraction was subjected to four additional centrifugations at 1,000 × *g* in a JS13.1 rotor for 7 min at 4ºC. The PNS was fractionated by centrifugation at 20,000 × *g* in a JS13.1 rotor for 35 min at 4ºC into pellet (20KgP) and supernatant (20KgS) fractions.

The 20KgP was resuspended in 11% (w/v) Nycodenz in buffer H and loaded onto the top of a discontinuous Nycodenz gradient (6.6 ml of 17%, 16.5 ml of 25%, 4.5 ml of 35% and 3 ml of 50% (w/v) Nycodenz in buffer H). Organelles were separated by ultracentrifugation at 100,000 × *g* for 90 min at 4ºC in a Beckman VTi50 rotor. 18 fractions of 2 ml each were collected from the bottom of the gradient.

To prepare 200KgP and 200KgS fractions, the 20KgS was collected and subjected to centrifugation at 200,000 × *g* for 45 min at 4°C in a Beckman 70Ti rotor. The 200KgP was resuspended in 11% Nycodenz and layered on top of a discontinuous gradient made up of 5 ml of each of 15%, 20%, 25%, 30%, 35%, 40% and 2 ml of 50% (w/v) Nycodenz in buffer H formed ~24 h in advance and kept at 4°C. Centrifugation was at 100,000 × *g* for 18 h at 4°C in a VTi50 rotor. 18 fractions of 2 ml each were collected from the bottom of the gradient. Fractions were subjected to TCA precipitation to concentrate protein and analyzed by immunoblotting.

2.7.2 Extraction and subfractionation of peroxisomes

Extraction and subfractionation of peroxisomes were performed according to Smith *et al*. (2000) with modifications. Essentially, organelles in the 20KgP fraction (containing ~50 µg of protein) were lysed by incubation in 10 volumes of ice-cold Ti8 buffer (10 mM Tris-HCl, pH 8.0) containing 2x complete protease inhibitor cocktail (Roche) on ice for 1 h with occasional vortexing and separated by ultracentrifugation at 200,000 × *g* for 1 h at 4ºC in a Beckman

TLA120.2 rotor into a membrane fraction (Ti8P) and a soluble fraction (Ti8S). The Ti8P fraction was resuspended in ice-cold Ti8 buffer to a final protein concentration of 0.5 mg/ml and mixed with 10 volumes of ice-cold 0.1 M Na_2CO_3, pH 11.3. The mixture was incubated on ice for 45 min with occasional vortexing and subjected to ultracentrifugation at 200,000 × *g* for 1 h at 4ºC in a TLA120.2 rotor to yield a fraction enriched for integral membrane proteins (CO_3P) and a fraction enriched for peripheral membrane proteins (CO_3S).

2.8 Microscopy

2.8.1 Confocal 3D video microscopy

Yeast strains expressing GFP and/or mRFP fusion proteins were grown to mid-log phase in YEPD medium or SM medium, and then for 8 h in YPBO medium or for 16 h in SCIM, if required. Images were captured and analyzed essentially as described (Fagarasanu *et al*., 2009) with modifications. Specifically, 2 µl of rinsed cells were combined with 8 µl of warmed nonfluorescent medium containing 1.5% low-melting agarose and spread on a slide with two 18-mm square wells (Cel-line Brand, Thermo Scientific, Waltham, MA). Cells were incubated at room temperature for image capture as described (Hammond and Glick, 2000) using a modified LSM 510 META confocal microscope equipped with a 63x 1.4 NA Plan-Apo objective (Carl Zeiss). A piezoelectric actuator was used to drive continuous objective movement, allowing for the rapid collection of *z*-stacks. Stacks of 37 optical sections spaced 0.16 µm apart were captured

Acquired images were deconvolved using algorithms provided by Huygens Professional Software (Scientific Volume Imaging, Hilversum, The Netherlands). For this method, three-dimensional (3D) data sets were processed to remove noise and reassign blur through an iterative Classic Maximum Likelihood Estimation algorithm and an experimentally derived point spread function.

A Gaussian filter was applied to the transmission image in Huygens, and blue color was applied to the transmission image using Imaris 7.0 software (Bitplane, South Windsor, CT). The level of the transmission image was modified, and the image was processed until only the circumference of the cell was visible. To prevent interference by internal structures captured in the transmission image, internal structures were removed in Adobe Photoshop. Imaris 7.0 was subsequently used to display the deconvolved 3D data set with the processed transmission image and to prepare the image files before final figure assembly in Adobe Photoshop and Adobe Illustrator. All images shown are representative, maximum intensity projections. Quantification was done using the surface measure function in Imaris 7.0.

2.8.2 Electron microscopy

Cells were processed for electron microscopy as described by Goodman *et al*. (1990). All microcentrifugations were at 16,000 × g for 1 min, and all incubations were done in 1.5-ml microcentrifuge tubes at room temperature with agitation, unless indicated otherwise. Cells were grown 16 h in SCIM, harvested and washed twice in 1% Brij 35. Approximately 100 µl of cell

pellet were fixed in 1 ml of 3% $KMnO_4$ for 15 min, washed twice with water, and incubated in 1 ml of 1% sodium periodate for 10 min. Cells were pelleted, washed once with water, and incubated with 1 ml of 1% NH_4Cl for 10 min. Cells were again pelleted, washed once with water, and subjected to serial dehydration in 60%, 80%, 95%, and 100% ethanol and in propylene oxide. Each incubation was for 5 min. Incubation in propylene oxide was repeated three times. Cells were collected and incubated in 1 ml of a 1:1 mixture of propylene oxide and resin (a mixture of TAAB 812 resin, specially distilled DDSA, and methyl nadic anhydride (MNA) in proportions suggested by the manufacturer (Canemco & Marivac)) for 1 h. Cells were next pelleted and resuspended in 1 ml of resin. Incubation with resin was carried out for 1 h with agitation and for 3 h in a fume hood with caps open. Finally, cells were harvested by microcentrifugation for 8 min, then resuspended in 1 ml resin containing 1% DMP-30. Cells were incubated in the dark overnight at room temperature with constant agitation. Cells were then centrifuged for 8 min, resuspended in resin with 2% DMP-30 and incubated at room temp with agitation for 2 h. Small portions of cells were transferred to embedding capsules (EMS) containing resin with 2% DMP-30. Embedding capsules were placed in an oven at 60°C to allow the resin to polymerize. 80-nm ultra-thin sections were cut using an Ultra-Cut E Microtome (Reichert-Jung), stained with 1% lead citrate and examined on a Phillips 410 electron microscope. Images were captured with a digital camera (Soft Imaging System) and subjected to morphometric analysis using iTEM software as previously described (Tam *et al*., 2003).

2.9 Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed using the Matchmaker Two-Hybrid System according to the manufacturer's instructions (Clontech) with modifications.

2.9.1 Construction of chimeric genes

Chimeric genes were made by amplifying the open reading frames (ORFs) of *PEX11*, *PEX25*, *PEX27*, *FIS1*, *VPS1* and *PEX34* by PCR and ligating them in-frame and downstream of sequences encoding the activation domain (AD) and DNA-binding domain (DB) of the GAL4 transcriptional activator in plasmids pGAD424 and pGBT9, respectively. All PCR products were digested with EcoRI, BamHI or PstI and ligated into pGAD424 and pGBT9.

2.9.2 Assays for two-hybrid interactions

Plasmid pairs encoding AD and DB fusion proteins were transformed into *S. cerevisiae* strain *SFY526* as described in Section 2.3.2. Transformants were grown in SM. Possible interaction between AD and DB fusion proteins were detected by testing for activation of the integrated *LacZ* construct using the β-galactosidase filter assay according to the instructions of Clontech. For filter assays, cells were streaked directly onto filter paper placed on solid medium, incubated at 30°C overnight, then broken by 4 freeze-thaw cycles at -80ºC. The filter paper was then laid on a second filter paper saturated with 10 ml of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$)

containing 27 µl of 2-mercaptoethanol and 167 µl X-gal (20 mg/ml in DMF stored at -20°C). Filters were sealed and incubated at room temperature in the dark until color developed.

CHAPTER 3

PEX34P IS A PEROXISOMAL MEMBRANE PROTEIN INVOLVED IN REGULATING THE PEROXISOME POPULATION IN *S. CEREVISIAE*

A version of this chapter has previously been published as "The Peroxin Pex34p functions with the Pex11 family of peroxisomal divisional proteins to regulate the peroxisome population in yeast" (Robert J. Tower, Andrei Fagarasanu, John D. Aitchison and Richard A. Rachubinski. 2011. *Molecular Biology of the Cell* 22:1727-1738). Reprinted with permission.

3.1 Overview

This chapter reports the identification of the *PEX34* gene encoding a novel peroxisomal integral membrane protein. Pex34p acts independently and also in concert with the Pex11 protein family members Pex11p, Pex25p, and Pex27p to control the peroxisome populations of cells under conditions of both peroxisome proliferation and constitutive peroxisome division. Yeast two-hybrid analysis showed that Pex34p interacts physically with itself and with Pex11p, Pex25p, Pex27p and Fis1p. Pex34p requires Pex11 family proteins to promote peroxisome division and can act as a positive effector of peroxisome division as its overexpression leads to increased numbers of peroxisomes in wild-type and *pex34*Δ cells. Our analysis of peroxisomes under conditions of noninduction have also identified a novel role for both Pex25p and Pex34p in peroxisome biogenesis and/or maturation and for Pex27p in a late acting stage of peroxisome division after peroxisomal elongation. Our discovery of Pex34p as a protein involved in the already complex control of peroxisome populations emphasizes the necessity of cells to strictly regulate their peroxisome populations to be able to respond appropriately to changing environmental conditions.

3.2 Pex34p is a peroxisomal integral membrane protein

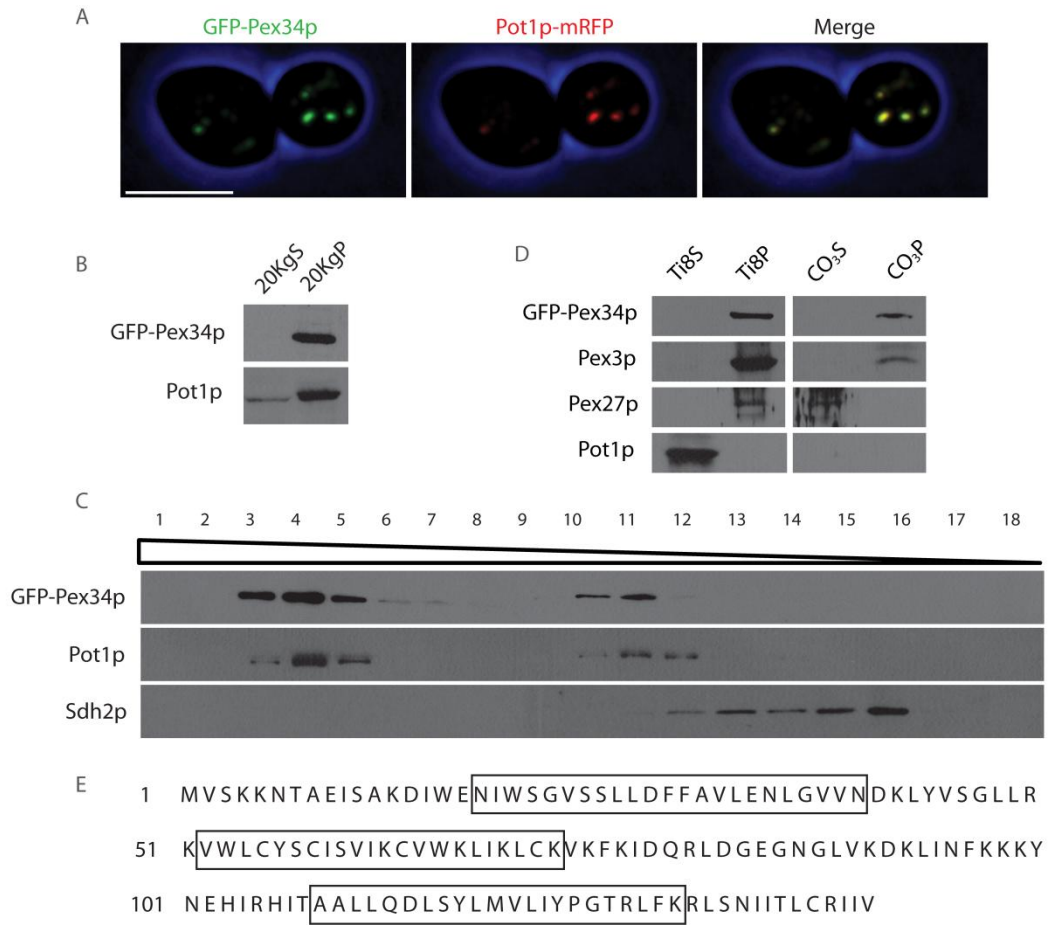
Large-scale protein interaction studies have provided evidence of interaction of the protein of unknown function encoded by the *S. cerevisiae* ORF *YCL056c* and a number of proteins involved in different aspects of peroxisome dynamics including Pex7p, Pex10p, Pex13p, Pex15p and Fis1p (Yu *et al.*, 2008;

Yeast Resource Center [http://www.yeastrc.org./]). In addition, a global analysis of protein localization in *S. cerevisiae* by fluorescence microscopy showed that a GFP-tagged version of the Ycl056c protein gave a punctate pattern of fluorescence similar to that exhibited by fluorescently labeled peroxisomes (Huh *et al.*, 2003). These findings prompted us to determine whether the Ycl056c protein is indeed peroxisomal and whether it has a role in peroxisome dynamics. Data presented herein demonstrate that the Ycl056c protein localizes to peroxisomes and has a role in peroxisome dynamics. Accordingly, we have designated it a peroxin, Pex34p, and its encoding gene *PEX34*. Phylogenetic analysis suggests homologues of Pex34p appear to be restricted to members of the *Saccharomycetaceae*, including *Kluveromyces, Zygosaccharomyces,* and other species of *Saccharomyces* (Byrne and Wolfe, 2005).

Pex34p tagged at its N-terminus with GFP (GFP-Pex34p) colocalized with Pot1p-mRFP, a fluorescent protein fusion between peroxisomal 3-ketoacyl-CoA thiolase (Pot1p) and monomeric red fluorescent protein (mRFP), to punctate structures characteristic of peroxisomes (Figure 3-1A). Subcellular fractionation was also used to establish that Pex34p is associated with peroxisomes. GFP-Pex34p, like the peroxisomal matrix protein Pot1p, localized preferentially to a 20,000 × *g* pellet (20KgP) fraction enriched for mature peroxisomes and some forms of immature peroxisomes (Tam *et al.*, 2003; Vizeacoumar *et al.*, 2003; 2004) (Figure 3-1B). Isopycnic density gradient centrifugation of the 20KgP fraction showed that GFP-Pex34p cofractionated with Pot1p but not with the mitochondrial protein Sdh2p (Figure 3-1C).

Organelle extraction was used to determine the suborganellar location of Pex34p. Organelles in the 20KgP fraction were subjected to hypotonic lysis in dilute alkali Tris buffer, followed by ultracentrifugation to yield a supernatant (Ti8S) fraction enriched for soluble proteins and a pellet (Ti8P) fraction enriched for membrane proteins (Figure 3-1D). GFP-Pex34p cofractionated with the peroxisomal integral membrane protein Pex3p and the peroxisomal peripheral membrane protein Pex27p to the Ti8P fraction, whereas the soluble peroxisomal matrix protein Pot1p was found almost exclusively in the Ti8S fraction. The Ti8P fraction was further extracted with alkali Na_2CO_3 and subjected to ultracentrifugation. This treatment releases proteins associated with, but not integral to, membranes (Fujiki *et al.*, 1982). GFP-Pex34p cofractionated with Pex3p to the pellet (CO_3P) fraction enriched for integral membrane proteins but not with Pex27p to the supernatant (CO_3S) fraction enriched for peripheral membrane proteins. These data suggest that Pex34p is an integral membrane protein of peroxisomes, consistent with the predictions of three topology prediction programs (SOSUI [http://bp.nuap.nagoya-u.ac.jp/sosui/], HMMTOP [http://www.enzim.hu/ hmmtop/], and TMpred [http://www.ch.embnet.org /software/TMPRED_form.html]) that Pex34p contains three transmembrane spanning regions (Figure 3-1E).

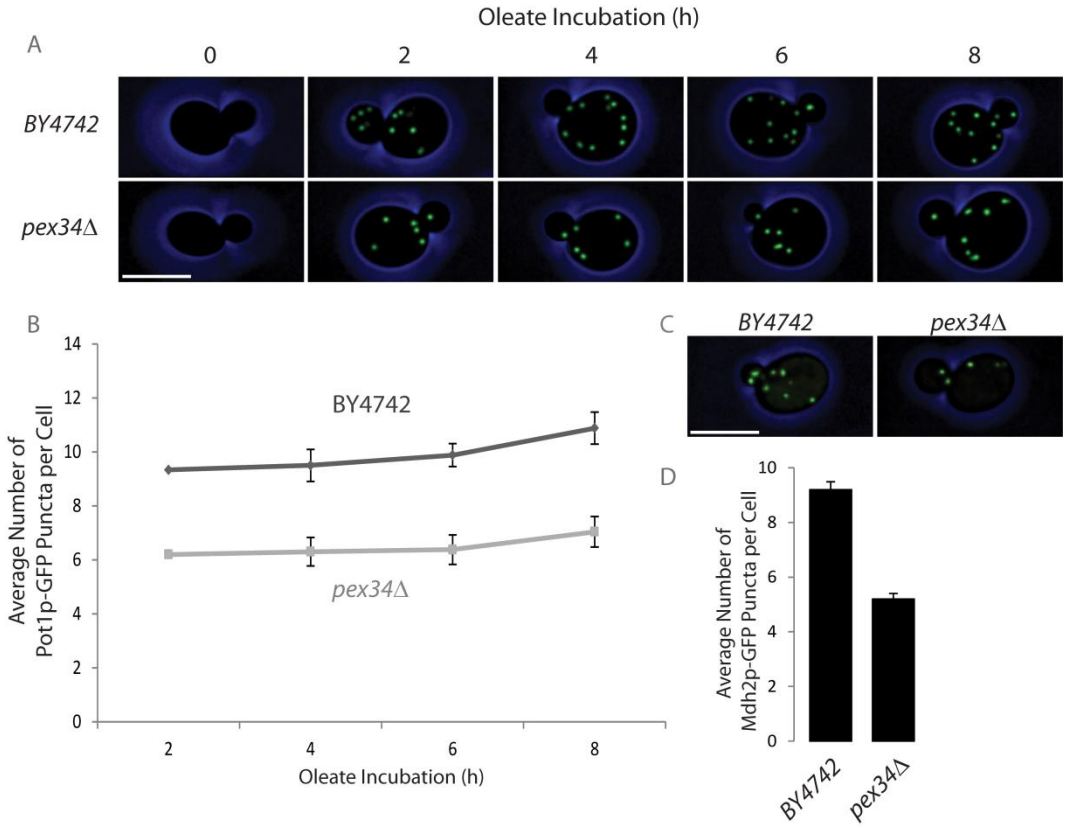
FIGURE 3-1: Pex34p is a peroxisomal integral membrane protein. (A) GFP-Pex34p colocalizes with the chimeric peroxisomal marker protein Pot1p-mRFP to punctate structures characteristic of peroxisomes by confocal fluorescence microscopy. Bar, 5 μm. (B) GFP-Pex34p localizes to the 20KgP subcellular fraction enriched for peroxisomes. Immunoblot analysis of equivalent portions of the 20KgS and 20KgP fractions from cells expressing GFP-Pex34p was performed with antibodies to GFP and to the peroxisomal matrix protein, Pot1p. (C) GFP-Pex34p cofractionates with peroxisomes. Organelles in the 20KgP fraction were separated by isopycnic centrifugation on a discontinuous Nycodenz gradient. Fractions were collected from the bottom of the gradient, and equal portions of each fraction were analyzed by immunoblotting. Fractions enriched for peroxisomes and mitochondria were identified by immunodetection of Pot1p and Sdh2p, respectively. (D) The 20KgP fraction from cells expressing GFP-Pex34p was treated with 10 mM Tris-HCl, pH 8.0, to lyse peroxisomes and was then subjected to ultracentrifugation to yield a supernatant (Ti8S) fraction enriched for matrix proteins and a pellet (Ti8P) fraction enriched for membrane proteins. The Ti8P fraction was treated further with 0.1 M Na_2CO_3, pH 11.3, and separated by ultracentrifugation into a supernatant (CO_3S) fraction enriched for peripheral membrane proteins and a pellet (CO_3P) fraction enriched for integral membrane proteins. Equal portions of each fraction were analyzed by immunoblotting with antibodies to GFP, the matrix protein Pot1p, the peroxisomal integral membrane protein Pex3p, and the peroxisomal peripheral membrane protein Pex27p. (E) Amino acid sequence of Pex34p. Boxed sequences designate three membrane-spanning regions predicted by SOSUI.


A
GFP-Pex34p
Pot1p-mRFP
Merge
B
20KgS
20KgP
GFP-Pex34p
Pot1p
D
Ti8S
Ti8P
CO3S
CO3P
GFP-Pex34p
Pex3p
Pex27p
Pot1p
C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
GFP-Pex34p
Pot1p
Sdh2p
E
1 MVSKKNTAEISAKDIWENIWSGVSSLLDFFAVLENLGVVNDKLYVSGLLR
51 KVWLCYSCISVIKCVWKLIKLCKVKFKIDQRLDGEGNGLVKDKLINFKKKY
101 NEHIRHITAALLQDLSYLMVLIYPGTRLFKRLSNIITLCRIIV

3.3 Deletion of the *PEX34* gene affects peroxisome abundance under conditions of both peroxisome proliferation and constitutive division

Wild-type and *pex34*Δ cells expressing oleic acid-inducible Pot1p-GFP were grown in glucose-containing medium and then transferred to YPBO medium containing oleic acid as the sole carbon source to induce peroxisome proliferation. Cells were imaged by confocal fluorescence microscopy every 2 h (Figure 3-2A), and the number of Pot1p-GFP-labeled peroxisomes per cell was quantified (Figure 3-2B). Cells deleted for the *PEX34* gene contained fewer peroxisomes than wild-type cells over the time of observation (up to 8 h). To determine whether this difference in peroxisome numbers between *pex34*Δ cells and wild-type cells was dependent on conditions promoting peroxisome proliferation, we analyzed *pex34*Δ cells and wild-type cells that constitutively express a chimera between GFP and the peroxisomal protein, malate dehydrogenase 2 (Mdh2p-GFP) (Huh *et al*., 2003; Wolinski *et al*., 2009), under conditions of constitutive peroxisome division, i.e. growth of cells in glucose-containing medium. *pex34*Δ cells continued to exhibit reduced numbers of peroxisomes compared with wild-type cells under conditions of constitutive peroxisome division (Figure 3-2C,D). Thus, Pex34p plays a role in maintaining the abundance of peroxisomes under conditions of both peroxisome proliferation and constitutive peroxisome division.

FIGURE 3-2: Cells deleted for the *PEX34* gene have reduced numbers of peroxisomes. (A and B) The wild-type strain *BY4742* and the deletion strain *pex34*Δ expressing Pot1p-GFP were grown in glucose-containing medium and then transferred to medium containing oleic acid as the sole carbon source to promote peroxisome proliferation. Fluorescent images of cells were captured by confocal microscopy every 2 h during oleic acid incubation (A) and scored for the number of Pot1p-GFP-labeled puncta per cell (B). Graphic results present the average number of puncta ± SEM of three independent experiments and 20 budded cells per experiment. (C and D) The wild-type strain *BY4742* and the deletion strain *pex34*Δ expressing Mdh2p-GFP were sampled during exponential growth in glucose-containing medium, imaged by confocal fluorescence microscopy (C), and scored for the number of Mdh2p-GFP-labeled puncta per cell (D). Graphic results present the average number of puncta ± SEM of three independent experiments and 20 cells per experiment. Bar represents 5 μm in panels (A) and (C).


A
Oleate Incubation (h)
0
2
4
6
8
BY4742
pex34Δ
B
Average Number of Pot1p-GFP Puncta per Cell
BY4742
pex34Δ
Oleate Incubation (h)
C
BY4742
pex34Δ
D
Average Number of Mdh2p-GFP Puncta per Cell
BY4742
pex34Δ

3.4 Pex34p interacts with proteins of the Pex11p family to control peroxisome morphology and abundance under conditions of peroxisome proliferation

A limited yeast two-hybrid screen was done between Pex34p and other proteins previously implicated in peroxisome division to determine potential physical interactions between them. Chimeric genes were constructed by fusing the ORFs of genes of interest in-frame and downstream of sequences encoding one of two functional domains, the transcription-activating domain (AD) and the DNA-binding domain (BD), of the Gal4p transcriptional activator. Pairwise combinations were transformed into *S. cerevisiae* strain *SFY526* and analyzed using a β-galactosidase filter detection assay (Figure 3-3A). As previously reported, Pex34p was found to interact with Fis1p (Yu *et al*., 2008), a protein involved in both mitochondrial fission (Mozdy *et al*., 2000) and peroxisome division (Koch *et al*., 2005; Motley *et al*., 2008), and also with Pex11p, Pex25p, and Pex27p, which together make up the Pex11 family of proteins controlling peroxisome proliferation (Erdmann and Blobel, 1995; Smith *et al*., 2002; Rottensteiner *et al*., 2003; Tam *et al*., 2003). These interactions were specific, as the AD and BD fusions showed no self-activation and no interaction between Pex34p and the dynamin-like protein Vps1p was detected which is known to play a role in peroxisome fission (Hoepfner *et al*., 2001).

Confocal fluorescence microscopy of *pex11*Δ, *pex25*Δ, and *pex27*Δ cells expressing Pot1p-GFP and grown in oleic acid-containing medium showed reduced numbers of peroxisomes compared with wild-type cells, as has been previously reported (Erdmann and Blobel, 1995; Smith *et al*., 2002; Rottensteiner

et al., 2003; Tam *et al*., 2003), and confirmed the reduction in peroxisome number in *pex34*Δ cells (Figure 3-3B,C). A greater reduction in the number of peroxisomes was observed in the double deletion strains *pex34*Δ*pex11*Δ and *pex34*Δ*pex27*Δ, but not in the *pex34*Δ*pex25*Δ strain, as compared with the individual *pex11*Δ, *pex27*Δ, and *pex25*Δ strains (Figure 3-3B,C).

Single and double deletion strains grown in oleic acid-containing medium were further analyzed by electron microscopy for both peroxisome abundance and size (Figure 3-4). Morphometric analysis (Figure 3-4B,C) of electron micrographs (Figure 3-4A) showed a reduced number of enlarged peroxisomes in *pex34*Δ cells as compared with wild-type cells, whereas *pex34*Δ*pex11*Δ and *pex34*Δ*pex27*Δ cells showed fewer and larger peroxisomes than did cells deleted for the individual genes. Interestingly, deletion of the *PEX34* gene in the *pex25*Δ background often resulted in single, greatly enlarged peroxisomes frequently observed in *pex25*Δ cells being replaced with clustered, smaller peroxisomes (Figure 3-4A).

Taken together, genetic and microscopic analyses showed that Pex34p individually and together with Pex11p, Pex25p, and Pex27p acts to regulate peroxisome number and size under conditions that promote peroxisome proliferation.

FIGURE 3-3: Pex34p acts by itself and together with the proteins of the Pex11 family to control peroxisome size and number under conditions of peroxisome proliferation. (A) β-galactosidase filter assay to test for interaction between Pex34p and Pex34p, Pex11p, Pex25p, Pex27p, Fis1p, and Vps1p by yeast two-hybrid analysis. Two independent transformants for each strain are shown. (B) Wild-type *BY4742* cells and cells of the *pex34*Δ, *pex11*Δ, *pex25*Δ, *pex27*Δ, *pex34*Δ*pex11*Δ, *pex34*Δ*pex25*Δ, and *pex34*Δ*pex27*Δ deletion strains expressing Pot1p-GFP were grown for 16 h in oleic acid-containing SCIM and imaged by confocal fluorescence microscopy. Bar, 5 μm. (C) Cells were scored for the number of Pot1p-GFP-labeled puncta per cell. Graphic results represent the average number of puncta ± SEM of three independent experiments and 20 cells per experiment.

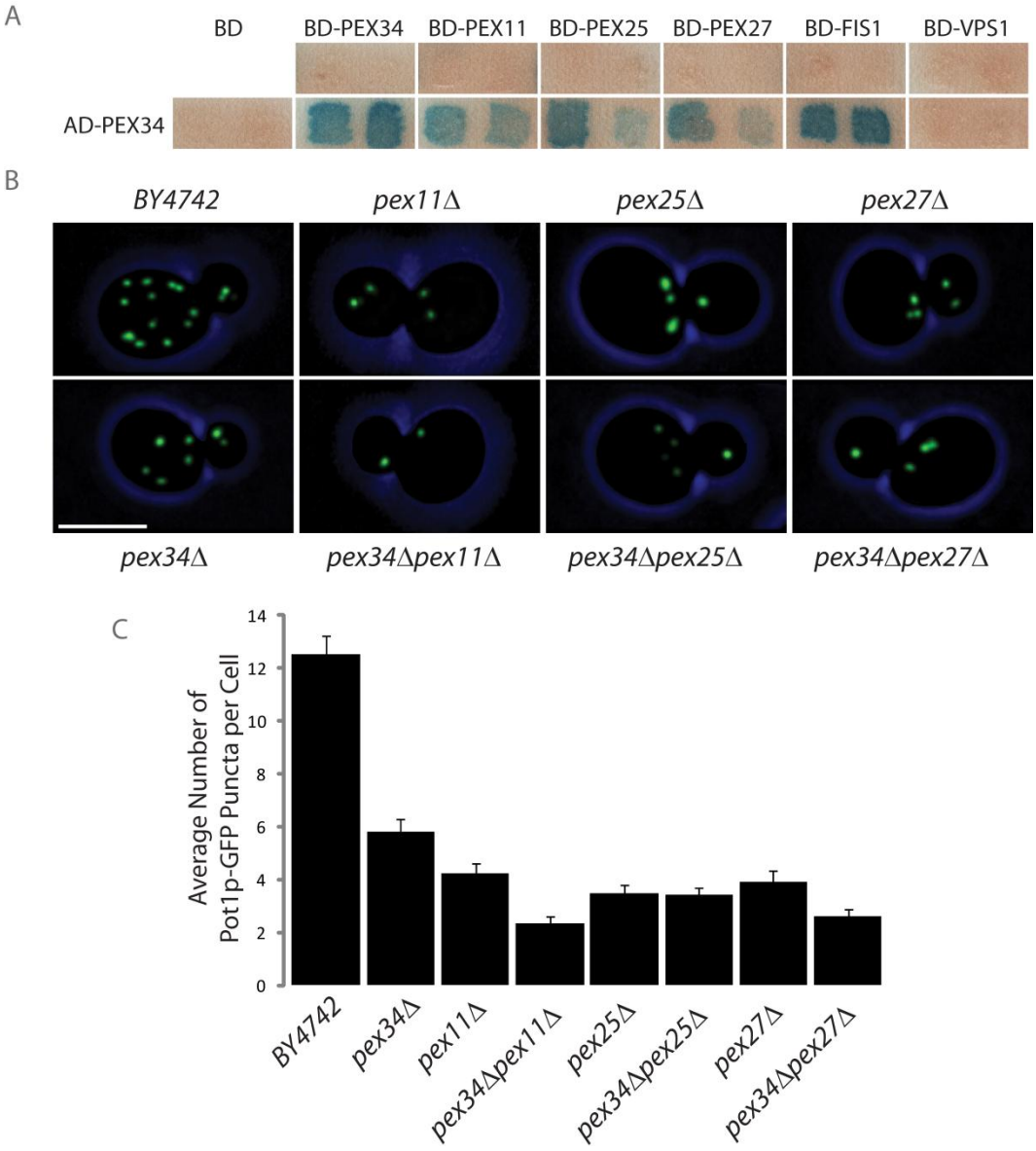
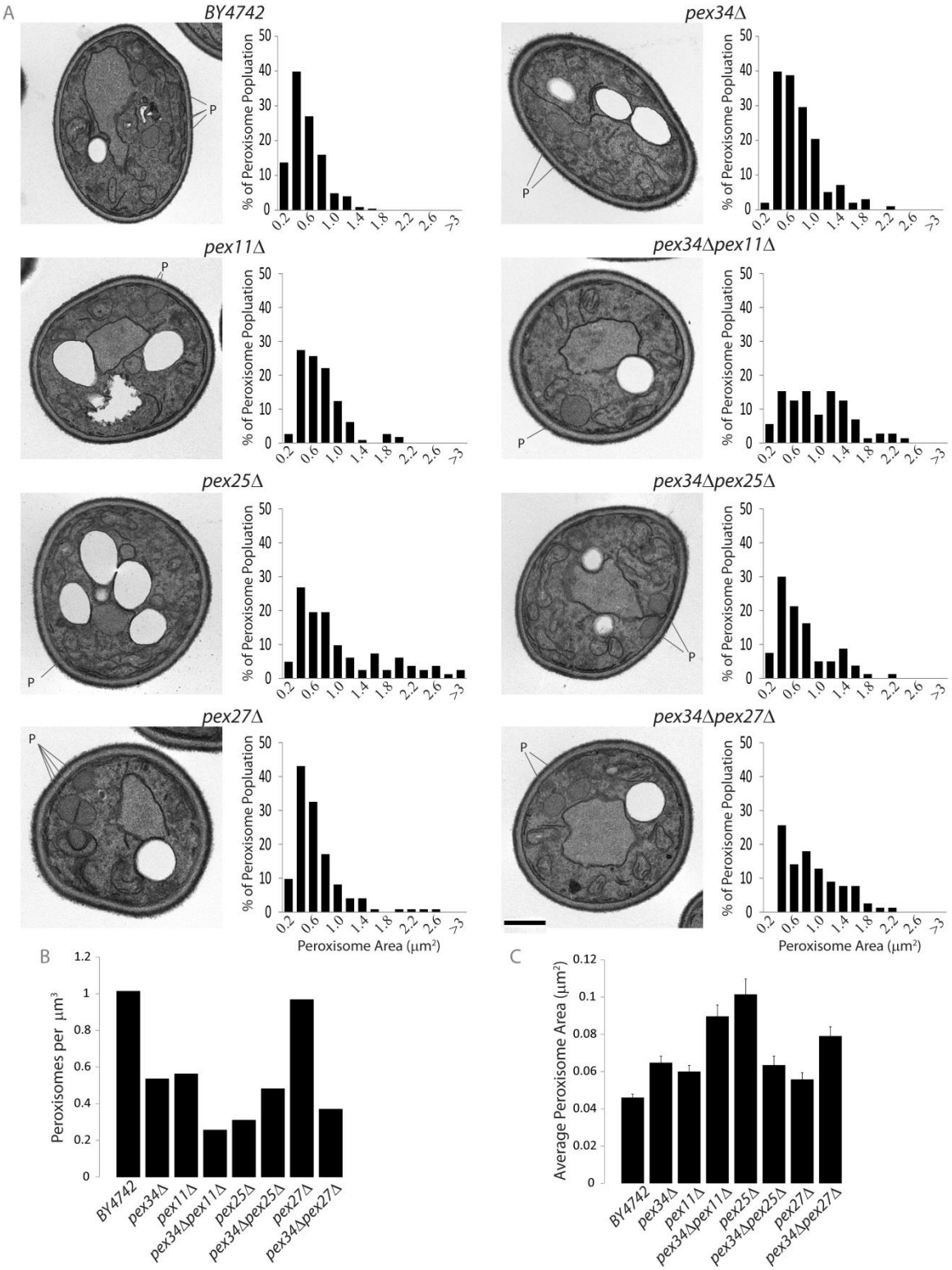

A
BD
BD-PEX34
BD-PEX11
BD-PEX25
BD-PEX27
BD-FIS1
BD-VPS1
AD-PEX34
B
BY4742
pex11Δ
pex25Δ
pex27Δ
pex34Δ
pex34Δpex11Δ
pex34Δpex25Δ
pex34Δpex27Δ
C
Average Number of Pot1p-GFP Puncta per Cell
0
2
4
6
8
10
12
14
BY4742
pex34Δ
pex11Δ
pex34Δpex11Δ
pex25Δ
pex34Δpex25Δ
pex27Δ
pex34Δpex27Δ

FIGURE 3-4: Deletion of the *PEX34* gene results in fewer and larger peroxisomes in oleic acid-grown cells. (A) Ultrastructure and morphometric analysis of cells of the wild-type strain *BY4742* and of different deletion strains. Cells were grown for 16 h in oleic acid-containing SCIM, fixed in 3% $KMnO_4$, and processed for electron microscopy. Bar, 1 μm. For morphometric analysis, the cell areas and areas of individual peroxisomes of 300 randomly selected cells from three independent analyses of each strain were determined using Olympus iTEM software. Peroxisomes were then separated into size categories, and a histogram depicting the percentage of total peroxisomes of each size category was generated for each strain. The numbers along the *x*-axis represent the maximum areas of peroxisomes (in square micrometers) for each category, with the exception of the last number, which represents the minimum area of peroxisomes (in square micrometers) in the last category. (B) Number of peroxisomes per cubic micrometer and (C) average peroxisome area (in square micrometers) for cells of the different strains. Error bars represent the SEM.


A
BY4742
pex34Δ
pex11Δ
pex34Δpex11Δ
pex25Δ
pex34Δpex25Δ
pex27Δ
pex34Δpex27Δ
P
% of Peroxisome Popluation
0 10 20 30 40 50
0.2 0.6 1.0 1.4 1.8 2.2 2.6 >3
Peroxisome Area (μm²)
B
Peroxisomes per μm³
0 0.2 0.4 0.6 0.8 1 1.2
BY4742
pex34Δ
pex11Δ
pex34Δpex11Δ
pex25Δ
pex34Δpex25Δ
pex27Δ
pex34Δpex27Δ
C
Average Peroxisome Area (μm²)
0 0.02 0.04 0.06 0.08 0.1 0.12

3.5 Pex34p interacts with proteins of the Pex11 family to control constitutive peroxisome division

Deletion strains expressing Mdh2p-GFP were grown in glucose-containing medium and were analyzed by confocal fluorescence microscopy to determine whether Pex34p acts together with members of the Pex11 protein family to also regulate peroxisome numbers under conditions of constitutive peroxisome division (Figure 3-5A). Like cells grown under conditions promoting peroxisome proliferation, deletion of the *PEX34* gene led to reduced numbers of peroxisomes as compared with wild-type cells (Figure 3-5B). Deletion of the *PEX25* and *PEX27* genes led to even greater reductions in the numbers of peroxisomes as compared with wild-type levels than did deletion of *PEX34* (Figure 3-5B), demonstrating a role for Pex25p and Pex27p in controlling peroxisome numbers under conditions of constitutive peroxisome division. Combining the deletion of *PEX34* with deletion of *PEX25* led to dramatic reductions in the numbers of peroxisomes per cell, whereas cells of the *pex34*Δ*pex27*Δ double deletion strain showed more modest, yet still significant, reductions in the number of peroxisomes when compared with cells deleted for *PEX27* alone (Figure 3-5B). Interestingly, both *pex27*Δ and *pex34*Δ*pex27*Δ cells often exhibited elongated vermiform peroxisomes (Figure 3-5A), similar to those previously described for cells lacking the dynamin-related protein, Vps1p (Hoepfner *et al*., 2001; Kuravi *et al*., 2006). This peroxisome phenotype was observed only in cells without Pex27p and only under conditions of constitutive peroxisome division.

Deletion of the *PEX11* gene did not lead to a dramatic reduction in the

number of peroxisomes compared with peroxisome numbers in wild-type cells under conditions of constitutive peroxisome division (Figure 3-5B). Expression of the *PEX11* gene is extremely low under conditions of cell growth in glucose but is greatly induced when cells are incubated in medium containing a carbon source like oleic acid that promotes peroxisome proliferation (Karpichev and Small, 1998; Smith *et al*., 2002; Knoblach and Rachubinski, 2010) and could possibly explain this observation. Deletion of *PEX34* in combination with *PEX11*, however, led to dramatically reduced numbers of peroxisomes, suggesting a role for Pex34p and the limited amounts of Pex11p present during glucose growth of cells in controlling peroxisome numbers during constitutive peroxisome division.

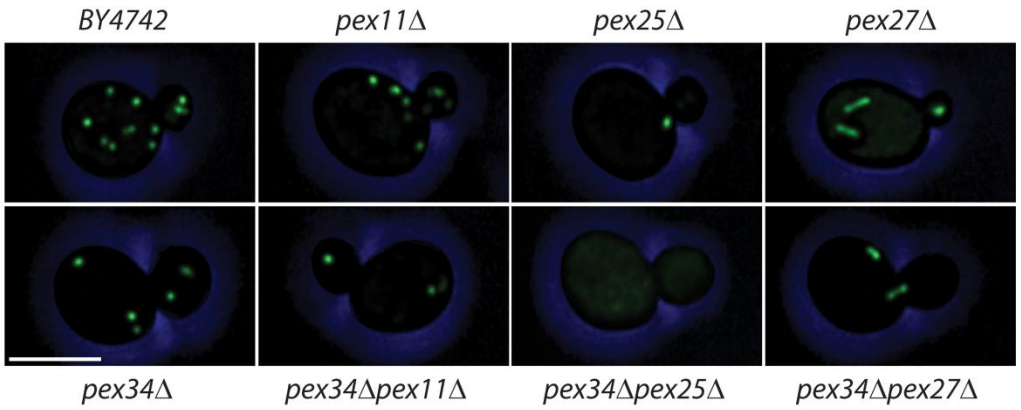
Together, our data demonstrate a role for Pex34p, alone and in conjunction with the Pex11p family of peroxisome divisional proteins, in controlling peroxisome numbers during both peroxisome proliferation and constitutive peroxisome division.

3.6 *PEX34* acts with *PEX25* to maintain mature peroxisomes in actively dividing cells

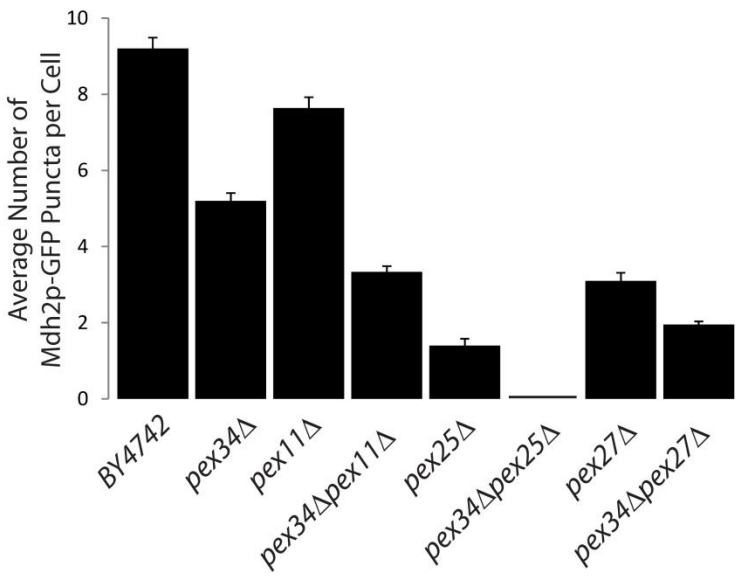
A significant proportion of *pex25*Δ cells (unpublished data) and all *pex34*Δ*pex25*Δ cells (Figure 3-5A) were observed to be devoid of Mdh2p-GFP-labeled puncta. Cells deleted for *PEX25* have been reported to be impaired in the import of PTS1-containing matrix proteins (Smith *et al*., 2002). Although Mdh2p does not contain a readily identifiable PTS1, its import into peroxisomes is dependent on the PTS1 receptor, Pex5p (unpublished data). We asked whether

FIGURE 3-5: Pex34p functions with the proteins of the Pex11 family to control peroxisome numbers under conditions of constitutive peroxisome division. (A) Wild-type *BY4742* cells and cells of different deletion strains expressing Mdh2p-GFP were harvested during exponential growth in glucose-containing YEPD medium and imaged by confocal fluorescence microscopy. Bar, 5 μm. (B) Cells were scored for the number of Mdh2p-GFP-labeled puncta per cell. Graphic results represent the average number of puncta ± SEM of three independent experiments and 20 cells per experiment.

A


BY4742
pex11Δ
pex25Δ
pex27Δ
pex34Δ
pex34Δpex11Δ
pex34Δpex25Δ
pex34Δpex27Δ

B

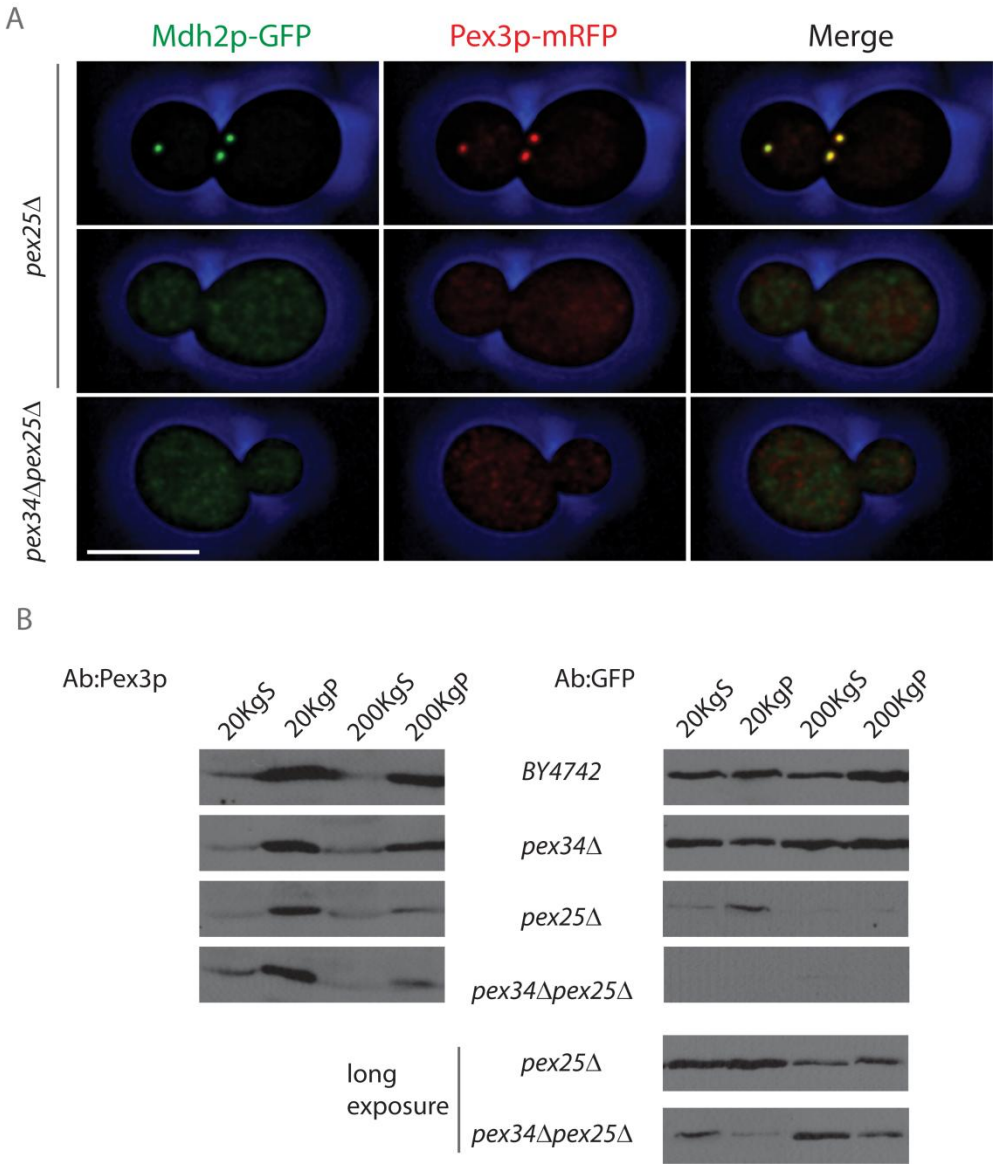

Average Number of Mdh2p-GFP Puncta per Cell
0
2
4
6
8
10
BY4742
pex34Δ
pex11Δ
pex34Δpex11Δ
pex25Δ
pex34Δpex25Δ
pex27Δ
pex34Δpex27Δ

*pex25*Δ cells and *pex34*Δ*pex25*Δ cells are truly devoid of peroxisomes or whether they are simply impaired in matrix protein import. *pex25*Δ cells and *pex34*Δ*pex25*Δ cells expressing Mdh2p-GFP were additionally labeled by a genomically expressed chimera of the peroxisomal membrane protein Pex3p and mRFP (Pex3p-mRFP) and grown in glucose-containing medium to permit robust cell growth. Cells were imaged by confocal fluorescence microscopy (Figure 3-6A). When present, Mdh2p-GFP-labeled puncta in *pex25*Δ cells colabeled with Pex3p-mRFP, whereas *pex34*Δ*pex25*Δ cells and *pex25*Δ cells lacking definitive Mdh2p-GFP-labeled puncta also lacked any definitive Pex3p-mRFP puncta, suggesting that a lack of Mdh2p-GFP puncta in cells with these genetic backgrounds is not simply the result of impaired matrix protein import but is due, at least in part, to compromised assembly of the peroxisomal membrane. Both Mdh2p-GFP and Pex3p-mRFP showed a generalized pattern of fluorescence in *pex34*Δ*pex25*Δ cells and in those *pex25*Δ cells lacking definitive puncta; neither chimeric protein exhibited preferential localization in the perinuclear region or at the cell periphery characteristic of an ER-localized protein.

To determine whether any of the Mdh2p-GFP pool in *pex34*Δ*pex25*Δ cells is present in membrane-bound compartments not visible by fluorescence microscopy, glucose-grown cells of the wild-type strain *BY4742* and of the deletion strains *pex34*Δ, *pex25*Δ, and *pex34*Δ*pex25*Δ were fractionated to yield 20KgS and 20KgP fractions. In addition, the 20KgS fraction was subjected to ultracentrifugation at 200,000 × *g* to yield a pellet (200KgP) fraction enriched for small vesicles and a cytosolic supernatant (200KgS) fraction. Equivalent portions

of the 20KgS and 20KgP and of the 200KgS and 200KgP were analyzed by immunoblotting with anti-Pex3p antibodies to detect Pex3p-mRFP and anti-GFP antibodies to detect Mdh2p-GFP (Figure 3-6B). In agreement with the results of fluorescence microscopy (Figure 3-6A), immunoblotting confirmed that Mdh2p-GFP was present in reduced amounts in *pex25*Δ and *pex34*Δ*pex25*Δ cells in comparison to wild-type *BY4742* or *pex34*Δ cells and required longer exposure for its ready detection (Figure 3-6B). In all strains, Pex3p-mRFP could be found in the 20KgP fraction, which contains both mature and some forms of immature peroxisomes (Tam *et al*., 2003; Vizeacoumar *et al*., 2003; 2004), and in the 200KgP fraction containing vesicular structures. Importantly, only small amounts of Mdh2p-GFP were present in the 20KgP fraction of *pex34*Δ*pex25*Δ cells as compared with cells of the wild-type and *pex34*Δ and *pex25*Δ strains, although some Mdh2p-GFP from *pex34*Δ*pex25*Δ cells could be found in the 200KgP fraction containing small vesicles. Therefore *pex34*Δ*pex25*Δ cannot readily assemble mature peroxisomes but can assemble vesicular structures containing the peroxisomal membrane marker protein chimera, Pex3p-mRFP. Our findings suggest that Pex34p acts in conjunction with Pex25p to maintain the population of mature peroxisomes in actively dividing cells.

FIGURE 3-6: *PEX34* and *PEX25* function in maintaining mature peroxisomes in actively dividing cells. (A) *pex25*Δ and *pex34*Δ*pex25*Δ cells expressing the fluorescent peroxisomal matrix protein chimera Mdh2p-GFP and the fluorescent peroxisomal membrane protein chimera Pex3p-mRFP were grown in glucose-containing medium to promote active cell division. Exponentially growing cells were imaged by confocal fluorescence microscopy. GFP and mRFP signal intensity in cells lacking peroxisomes has been increased to demonstrate a lack of discrete localization within the cell. Bar, 5 μm. (B) Cells of the wild-type *BY4742* strain and of the *pex34*Δ, *pex25*Δ, and *pex34*Δ*pex25*Δ deletion strains expressing Mdh2p-GFP and Pex3p-mRFP were grown in glucose-containing medium, harvested during exponential growth, and subjected to subcellular fractionation to yield 20KgS and 20KgP fractions. The 20KgS fraction was subjected to ultracentrifugation at 200,000 × *g* to yield a cytosolic 200KgS fraction and a 200KgP fraction containing small vesicles. Equivalent portions of each fraction were analyzed by immunoblotting with antibodies to Pex3p and GFP. The bottom two panels at right are a longer exposure of the corresponding *pex25*Δ and *pex34*Δ*pex25*Δ panels (top).


A
Mdh2p-GFP
Pex3p-mRFP
Merge
pex25Δ
pex34Δpex25Δ
B
Ab:Pex3p
20KgS
20KgP
200KgS
200KgP
Ab:GFP
20KgS
20KgP
200KgS
200KgP
BY4742
pex34Δ
pex25Δ
pex34Δpex25Δ
long exposure
pex25Δ
pex34Δpex25Δ

3.7 Deletion of *PEX27* results in elongated peroxisomes independent of peroxisomal inheritance machinery in rapidly dividing cells

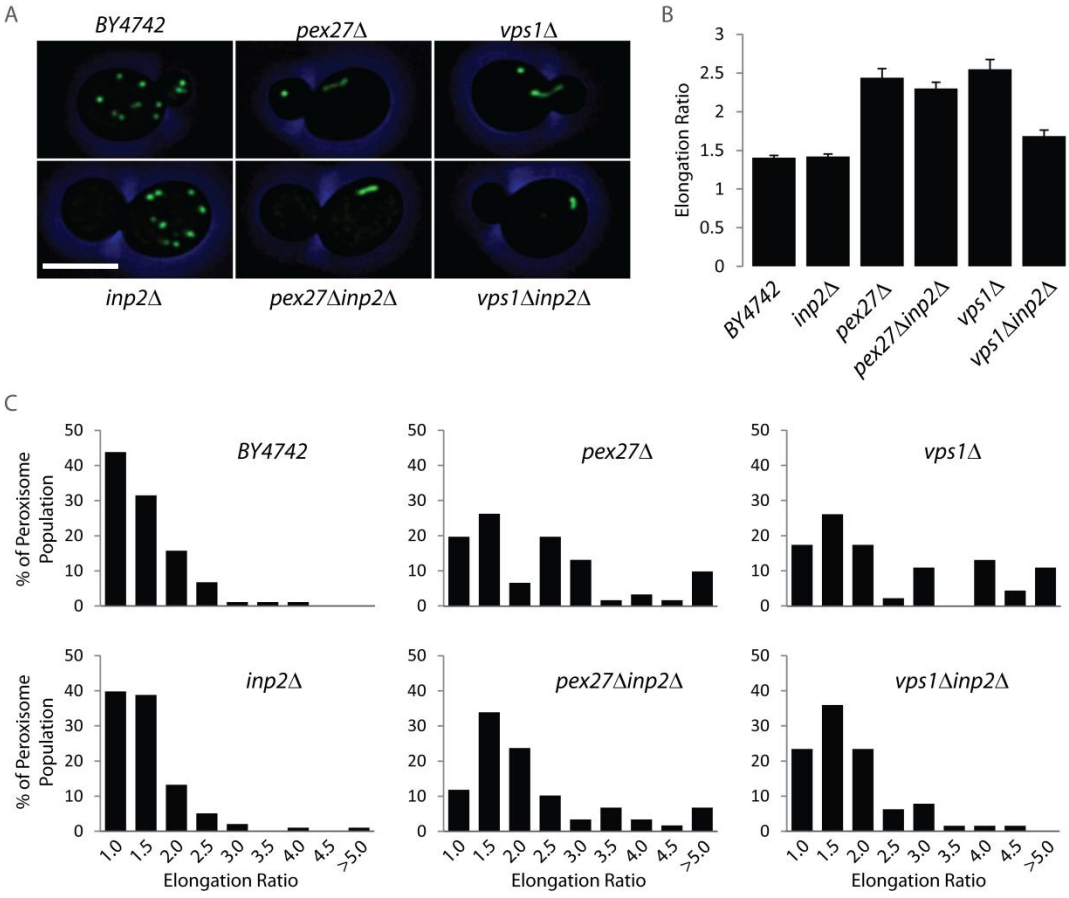
An increased frequency of elongated fluorescent structures was observed in *pex27*Δ cells compared to that of wild-type, *pex11*Δ, *pex25*Δ or *pex34*Δ. This observation is reminiscent of cells deficient for the dynamin-related protein Vps1p which typically contain a single enlarged peroxisome (Hoepfner *et al*., 2001). This peroxisome becomes elongated in response to the pulling force applied by myosin motors during the process of peroxisome inheritance. Deletion of the peroxisome-specific myosin motor adaptor Inp2p results in a disruption of peroxisome inheritance and a collapse of this elongated structure in *vps1*Δ cells (Fagarasanu *et al*., 2009). To determine whether the elongated peroxisomes observed in *pex27*Δ cells were the result of the peroxisome inheritance machinery or whether these structures represented a divisional intermediate independent of the pulling force applied by myosin motors, wild-type, *pex27*Δ and *vps1*Δ cells expressing the peroxisomal marker Mdh2p-GFP were additionally deleted for the *INP2* gene. Strains were grown in glucose-containing medium and imaged by confocal fluorescent microscopy (Figure 3-7A). Elongation ratios for each strain were determined by quantifying the ratio of the long axis to short axis dimensions for each peroxisome. Deletion of *VPS1* or *PEX27* resulted in a significant increase in the average elongation ratio of peroxisomes (Figure 3-7B) and an increased percentage of the peroxisome population existing as highly elongated structures, i.e. an elongation ratio >2) (Figure 3-7C) as compared to wild-type. While these elongated peroxisomes in *vps1*Δ cells were no longer present in the absence of

peroxisome inheritance and thus dependent on Inp2p (Hoepfner *et al*., 2001; Fagarasanu *et al*., 2009), elongated peroxisomes persisted in the *pex27*Δ*inp2*Δ double deletion strain. This suggests that the elongated structures present in *pex27*Δ cells are independent of the pulling force applied by the inheritance machinery and that this elongated structure may represent a divisional intermediate, with Pex27p normally functioning downstream of this elongation step in the division process.

3.8 Epistatic analysis of Pex34p and Pex11 protein family members in peroxisome division

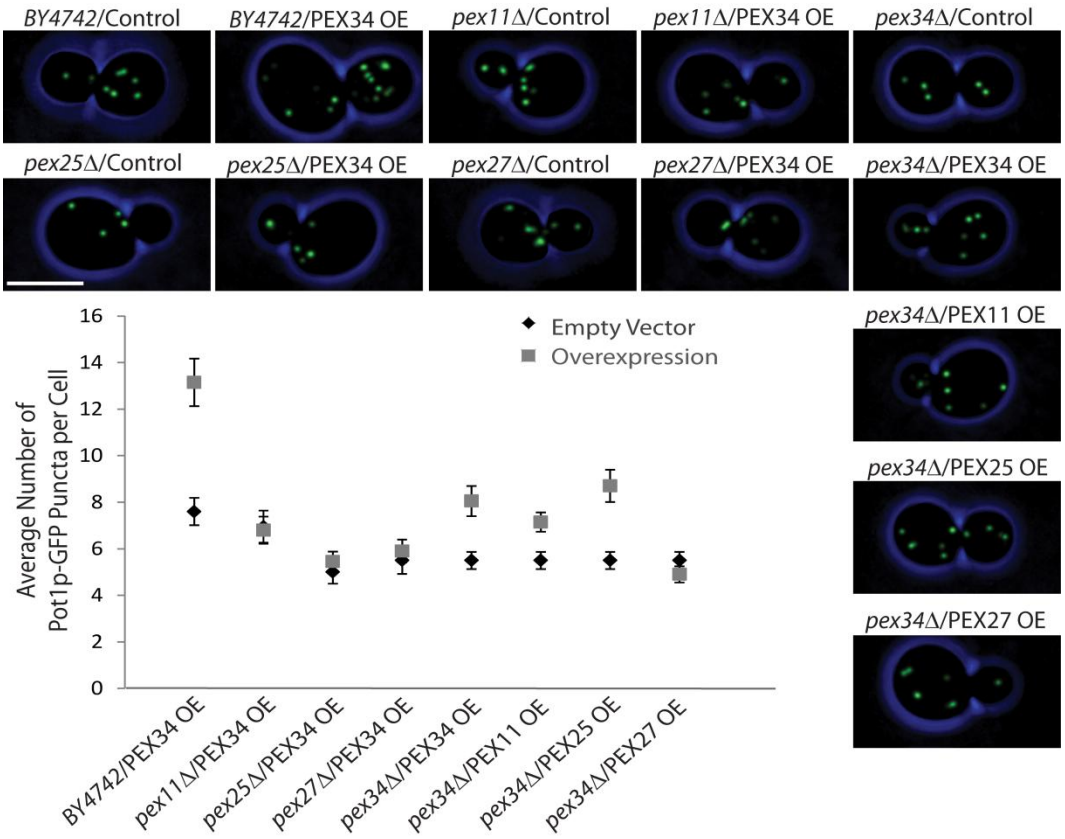
An epistatic analysis was done to investigate the interplay of *PEX34* and the genes of the *PEX11* family in peroxisome division. Plasmids expressing *PEX11*, *PEX25*, *PEX27*, or *PEX34* under the regulation of the galactose-inducible *GAL1* promoter were introduced into cells deleted for *PEX34* or for a gene of the *PEX11* family and containing Pot1p-GFP to fluorescently label peroxisomes. Cells carrying the empty pBY011 parental vector served as controls. Cells were first grown in oleic acid-containing medium, and galactose was then added to induce gene overexpression. Cells were imaged by confocal fluorescence microscopy, and GFP puncta were quantified (Figure 3-8). Wild-type cells or *pex34*Δ cells, but not *pex11*Δ, *pex25*Δ, or *pex27*Δ cells, overexpressing *PEX34* showed greater numbers of Pot1p-GFP puncta compared with their corresponding empty vector controls. These data suggest that Pex34p acts as a positive factor of peroxisome division and requires members of the Pex11 protein family to

Figure 3-7: *pex27*Δ cells contain elongated peroxisomes that form independently of the inheritance machinery. (A) Strains expressing the peroxisomal marker Mdh2p-GFP were grown in glucose-containing medium to exponential growth phase and imaged by confocal fluorescent microscopy for elongated peroxisomal structures. *vps1*Δ cells display highly elongated structures that collapse in the absence of Inp2p, while *pex27*Δ cells display elongated structures independently of Inp2p-mediated peroxisome inheritance. Confocal images were used to quantify each peroxisome's long and short axis dimensions to determine the peroxisomal elongation ration (long axis/short axis). Bar, 5 μm. Elongation ratios for each peroxisome were averaged for each strain (B) or separated into elongation ratio categories, and a histogram depicting the percentage of total peroxisomes of each size category was generated. The numbers along the *x*-axis represent the maximum elongation ratio of peroxisomes for each category, with the exception of the last number, which represents the minimum elongation ratio of peroxisomes in the last category (C). Error bars represent the SEM of three independent experiments and 20 cells per experiment.


A
BY4742
pex27Δ
vps1Δ
inp2Δ
pex27Δinp2Δ
vps1Δinp2Δ
B
Elongation Ratio
0
0.5
1
1.5
2
2.5
3
BY4742
inp2Δ
pex27Δ
pex27Δinp2Δ
vps1Δ
vps1Δinp2Δ
C
% of Peroxisome Population
BY4742
pex27Δ
vps1Δ
inp2Δ
pex27Δinp2Δ
vps1Δinp2Δ
0
10
20
30
40
50
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
>5.0
Elongation Ratio

promote peroxisome division. Moreover, overproduction of Pex11p or Pex25p could rescue the abnormal peroxisome phenotype of *pex34*Δ cells, reestablishing essentially wild-type levels of peroxisomes in these cells. In contrast, overproduction of Pex27p could not substitute for a lack of Pex34p in cells, as peroxisome numbers remained unchanged from what was observed in *pex34*Δ cells containing the empty pBY011 vector.

FIGURE 3-8: Pex34p acts as a positive effector of peroxisome division. *PEX34*, *PEX11*, *PEX25*, and *PEX27* were overexpressed from a galactose-inducible promoter in wild-type *BY4742* and mutant *pex34*Δ, *pex11*Δ, *pex25*Δ, and *pex27*Δ cells grown in oleic acid-containing medium and expressing the peroxisomal marker Pot1p-GFP. The number of GFP puncta were scored and plotted against cells carrying the empty expression vector pBY011 as a control. Values are the average number of puncta ± SEM of three independent experiments and 20 cells per experiment. Bar, 5 μm.


BY4742/Control
BY4742/PEX34 OE
pex11Δ/Control
pex11Δ/PEX34 OE
pex34Δ/Control
pex25Δ/Control
pex25Δ/PEX34 OE
pex27Δ/Control
pex27Δ/PEX34 OE
pex34Δ/PEX34 OE
pex34Δ/PEX11 OE
pex34Δ/PEX25 OE
pex34Δ/PEX27 OE
Empty Vector
Overexpression
Average Number of Pot1p-GFP Puncta per Cell
0
2
4
6
8
10
12
14
16
BY4742/PEX34 OE
pex11Δ/PEX34 OE
pex25Δ/PEX34 OE
pex27Δ/PEX34 OE
pex34Δ/PEX34 OE
pex34Δ/PEX11 OE
pex34Δ/PEX25 OE
pex34Δ/PEX27 OE

CHAPTER 4

DISCUSSION

4.1 Pex34p is a novel peroxisomal protein that functions alone or in concert with the Pex11 family of proteins to regulate the peroxisome population in *S. cerevisiae*

Global studies of protein-protein interactions have become key resources for predicting the possible functions of uncharacterized proteins in *S. cerevisiae* through their interactions with proteins of known biological function. These studies have demonstrated interactions between the uncharacterized protein encoded by the ORF *YCL056c*, which we have designated as Pex34p, and peroxins required for peroxisome biogenesis. Pex34p is a peroxisomal integral membrane protein that functions in controlling peroxisome abundance. Pex34p works in concert with the three members of the Pex11 protein family of peroxisome divisional factors, Pex11p, Pex25p and Pex27p, to control peroxisome abundance. Pex34p's primary role appears to be the regulation of the peroxisome population under conditions of noninduction of peroxisomes. *PEX34* mRNA levels are significantly reduced in response to growth in medium with oleate as the sole carbon source, the metabolism of which requires functional peroxisomes (Smith *et al*., 2002). Pex34p appears to be regulated also at the protein level, as detection of Pex34p by both confocal fluorescence microscopy and immunoblotting required the presence of glucose in the growth medium, even when *PEX34* expression was under control of the highly inducible galactose promoter. Cells deleted for the *PEX34* gene exhibit fewer peroxisomes under conditions of both peroxisome proliferation and constitutive peroxisome division. During growth in oleic acid-containing medium, which promotes peroxisome

proliferation, *pex34*Δ cells were observed by electron microscopy to have larger peroxisomes than did wild-type cells. No firm conclusion on the sizes of peroxisomes in *pex34*Δ cells grown in glucose-containing medium, in which peroxisomes divide constitutively, could be made by electron microscopy because peroxisomes under these conditions do not exhibit the characteristic "peroxisome morphology".

Pex34p interacts with itself and with Pex11p, Pex25p and Pex27p, implicating Pex34p homo- and hetero-oligomerization with Pex11 protein family members in regulating peroxisome division. Cells deleted for *PEX34* and for either *PEX11* or *PEX27* showed fewer peroxisomes than cells deleted individually for the genes under conditions of both peroxisome proliferation and constitutive peroxisome division. Under conditions of peroxisome proliferation, peroxisomes in *pex34*Δ*pex11*Δ cells and *pex34*Δ*pex27*Δ cells were larger than the peroxisomes in cells deleted for only one of the genes. Pex11p, Pex25p and Pex27p have been reported to act as positive effectors of peroxisome division, as their overproduction leads to increased numbers of peroxisomes in cells (Rottensteiner *et al.*, 2003; Tam *et al.*, 2003). Similarly, Pex34p acts as a positive effector of peroxisome division, as its overproduction leads to increased numbers of peroxisomes in wild-type and *pex34*Δ cells. However, Pex34p requires members of the Pex11 protein family to function as a positive effector of peroxisome division, suggesting that Pex34p may act at an early stage of peroxisome division.

Nevertheless, our results demonstrate that Pex34p, either alone or with members of the *PEX11* gene family, functions in controlling peroxisome abundance in cells that are proliferating peroxisomes in response to the presence of a carbon source requiring peroxisomes for its metabolism or are dividing peroxisomes constitutively to respond to the rapid cell division that occurs in a rich glucose-containing medium.

4.2 Peroxisome proliferation versus constitutive peroxisome division

Growth of yeast in a fatty acid-containing medium causes an expansion of the peroxisomal compartment and the transcriptional up-regulation of several genes involved in peroxisome dynamics and function (Thoms and Erdmann, 2005). Traditionally, studies of peroxisomes made use of these observations and used cells grown in oleic acid-containing medium to enhance their requirements for peroxisomes and, as a result, to enhance the observable phenotypes of cells associated with the absence of genes involved in peroxisome division (Rottensteiner *et al*., 2003; Vizeacoumar *et al*., 2003; 2006; Tam *et al*, 2003). Under these conditions, members of the Pex11 family have been shown to play major roles in peroxisome proliferation (Erdmann and Blobel, 1995; Smith *et al.*, 2002; Rottensteiner *et al.*, 2003; Tam *et al.*, 2003). Using the constitutively expressed peroxisomal marker Mdh2p-GFP, we have been able to observe subtle differences between peroxisome proliferation and constitutive peroxisome division and to identify those peroxins that play universal roles in peroxisome division and those that act specifically in one or the other type of division.

Unlike deletion of the *PEX34*, *PEX25* or *PEX27* gene, deletion of *PEX11* was found to affect peroxisome abundance only under conditions of peroxisome proliferation. The levels of Pex11p have been shown to be extremely low in glucose-grown cells and become elevated only when cells are grown in oleic acid-containing medium promoting peroxisome proliferation (Karpichev and Small, 1998; Smith *et al.*, 2002; Knoblach and Rachubinski, 2010). Therefore, cells have adapted to maintain their peroxisome number during rapid cell division with little requirement for Pex11p. Previous work has implicated Pex11p in the process of peroxisome elongation (Koch *et al*., 2010; Opalinski *et al*., 2011). It is interesting to speculate that under conditions of constitutive peroxisome division, the proposed peroxisome elongation function of Pex11p (Schrader *et al.*, 1998; Thoms and Erdmann, 2005; Koch *et al.*, 2010) may be substituted for, at least in part, by the pulling force applied by the inheritance machinery that has been shown to elongate peroxisomes in cells lacking Vps1p (Hoepfner *et al.*, 2001; Fagarasanu *et al.*, 2009).

Another unanswered question of peroxisome division is what mechanism triggers peroxisome division during conditions of peroxisome noninduction. In *Y. lipolytica*, peroxisome division has been shown to be initiated in response to the accumulation of matrix proteins, which results in remodeling of the peroxisomal membrane (Guo *et al*., 2007). During rapid cell division, peroxisomes must begin dividing to maintain the peroxisome population in the absence of extensive matrix protein import. Pex34p may function as a potential regulator of the constitutive peroxisome division pathway for several reasons: i) unlike most peroxisomal

genes, the mRNA encoding Pex34p is enriched in cells grown in glucose-containing medium, which supports robust cell division, compared to growth in oleic acid-containing medium, in which cell division is retarded; ii) epistatic analysis demonstrates that Pex34p is a positive regulator of peroxisome division but requires the Pex11 family of proteins for this function, which suggests an upstream or indirect rather than a direct role for Pex34p in peroxisome division; and iii) Pex34p has been shown to interact with several components of the peroxisomal matrix protein import machinery, suggesting a possible link between peroxisomal protein import and peroxisome division. Further work will be needed to determine how Pex34p effects peroxisome division, and to elucidate any functional links between Pex34p and components of the matrix protein import machinery.

4.3 Pex34p and Pex25p are involved in maintaining the population of mature peroxisomes in rapidly dividing cells

Deletion of *PEX34* and *PEX25*, but not *PEX34* and *PEX11* or *PEX27*, results in an increased number of small peroxisomes under conditions of peroxisome proliferation. Electron microscopy also showed that under conditions of peroxisome proliferation, *pex34*Δ*pex25*Δ cells contain more and smaller peroxisomes than do *pex34*Δ or *pex25*Δ cells. When grown in glucose-containing medium to promote constitutive peroxisome division, *pex34*Δ*pex25*Δ cells showed no characteristic peroxisomes by fluorescence microscopy. Mdh2p-GFP and Pex3p-mRFP did not form discrete, punctate foci but instead produced a

generalized fluorescence throughout these cells. Subcellular fractionation confirmed that these cells do not have mature peroxisomes but do have structures that are pelletable at high centrifugal forces and contain peroxisomal membrane and matrix proteins. At present, it is unknown whether these structures are some form of immature peroxisome or are *bona fide* functional peroxisomes that are uncharacteristically small. Our data suggest that Pex34p could have a role in peroxisome biogenesis outside its role in peroxisome division, as has been postulated for proteins of the *PEX11* family (Rottensteiner *et al.*, 2003). Because Pex34p is an integral membrane protein, it is possible that Pex34p traffics through the ER like many other peroxisomal membrane peroxins (van der Zand *et al*., 2010). After exiting the ER, Pex34p may recruit Pex25p and/or regulate its function. Pex25p, together with its partner Rho1p (Smith *et al.*, 2002), has been shown to have a role in the re-establishment of peroxisomes in peroxisome-deficient cells (Saraya *et al*., 2011).

4.4 Pex27p acts at a late stage in peroxisome division downstream of peroxisome elongation

Cells deleted for the *PEX27* gene show a high proportion of elongated peroxisomes under conditions of constitutive peroxisome division. Elongated peroxisomes were previously observed in cells lacking the dynamin-related protein, Vps1p, in which the myosin motor-dependent inheritance of the single enlarged peroxisome present in these cells results in elongation of the peroxisome into a tubular structure that passes through the neck between mother cell and bud

(Hoepfner *et al.*, 2001; Kuravi *et al.*, 2006; Fagarasanu *et al.*, 2009). We have demonstrated that the peroxisome elongation observed in *pex27*Δ cells is not the result of Inp2p-mediated pulling forces and instead may represent a normal intermediate in peroxisome division. This observation suggests that Pex27p may act downstream of peroxisome elongation, whereas Pex34p, Pex11p and Pex25p act upstream of or at this elongation step. Although electron microscopy analysis was not possible, confocal fluorescence microscopy suggested the structures observed in *pex27*Δ cells are tubular in nature in contrast to the structures in *vps1*Δ cells which exhibited constrictions, giving a "beads on a string" appearance (Hoepfner *et al*., 2001). Our data suggest a role for Pex27p in the constriction process, either directly or indirectly through the activation or recruitment of additional components, prior to Vps1p-mediated scission.

4.5 Synopsis and future directions

With the identification and initial characterization of Pex34p as a novel factor in the peroxisome divisional machinery and the elucidation that not all components of this divisional machinery are necessary for both peroxisome proliferation and constitutive division, a few important questions remain. Does Pex34p function depend directly on members of the Pex11p family or are they simply downstream effectors of a yet unidentified target for Pex34p? Is the process of constitutive peroxisome division linked to the cell cycle, and if so, how is this process regulated? Does Pex34p interact functionally with components of

the peroxisomal protein import machinery and are these interactions important for peroxisome division initiation under conditions of noninduction of peroxisomes?

Pex11p, Pex25p, Pex27p and Pex34p have all been shown to interact with themselves and each other (Tam *et al*., 2003; Rottensteiner *et al*., 2003). Furthermore, homodimerization of Pex11p has been shown to regulate its ability to proliferate peroxisomes under conditions of peroxisome induction (Marshall *et al*., 1996). These data suggest that homo- and heterodimerization between Pex11 family proteins and Pex34p may be important in regulating peroxisome division. Use of methods such as tandem-affinity purification (TAP) (Rigaut *et al*., 1999; Puig *et al*., 2001) of these proteins under conditions of both peroxisome induction and noninduction may provide greater insight into the complexes formed during peroxisome proliferation and constitutive division. Further analysis of these complexes in *pex34*Δ cells or cells in which Pex34p is overproduced may also provide insight into the role Pex34p has on modulating the formation of these protein complexes.

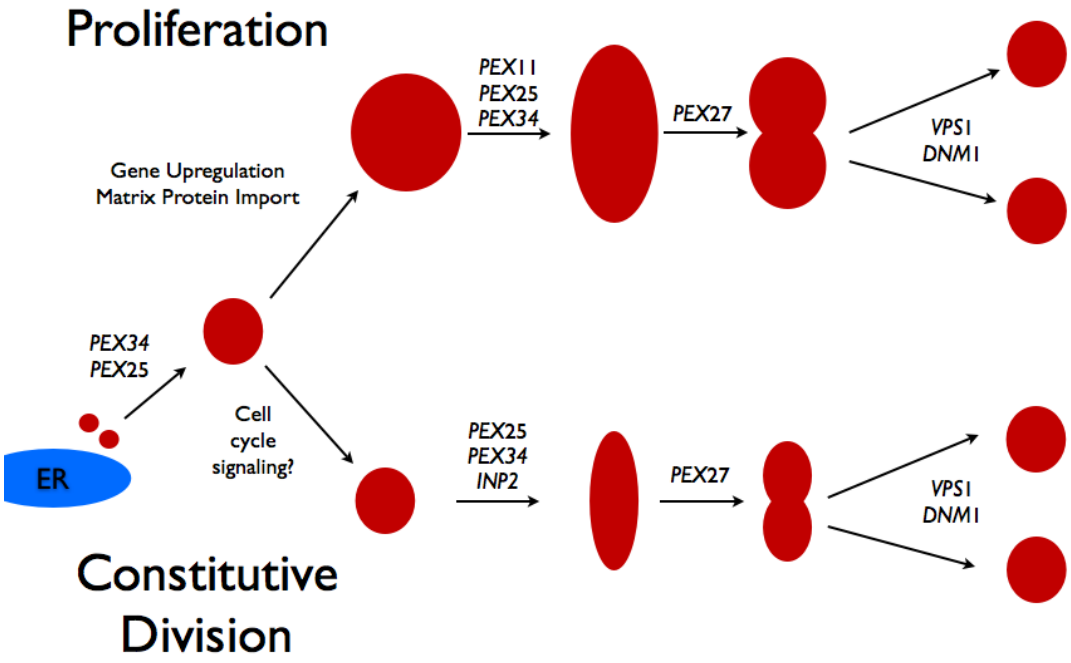
One way of linking cell division with peroxisome division would be to have a peroxin involved in peroxisome division to be cell cycle-regulated. Is Pex34p this peroxin? Although Pex34p levels during the cell cycle have not been determined, Pex34p mRNA levels do not appear to vary during the cell cycle (http://ww.yeastgenome.org/). Pex34p therefore probably does not link peroxisome division to cell division, and another protein or set of proteins working via a yet undefined mechanism accomplishes this important cross-talk between peroxisome dynamics and cell dynamics.

Pex34p has been shown to interact with several peroxins involved in the import of peroxisomal matrix proteins. Again using methods such as TAP, we could determine whether these interactions occur *in vivo* and whether the association of the different peroxins is in any way influenced by conditions of cell growth, *i.e.* conditions of peroxisome induction or noninduction. If Pex34p is involved in initiating peroxisome division in the absence of peroxisomal matrix protein accumulation within peroxisomes, it may be possible to rescue the *pex34*Δ phenotype by artificially overexpressing matrix proteins in cells under conditions of glucose growth. Overproducing matrix proteins in cells grown in glucose might restore peroxisome number in the absence of Pex34p by triggering a peroxisome proliferation-like divisional mechanism. Conversely, it would be of interest to observe the peroxisome population under conditions of induction in which peroxisomal matrix protein import is inhibited. Could overproduction of Pex34p initiate peroxisome division under conditions of peroxisome induction in the absence of matrix protein accumulation? Also it would be interesting to determine the effects of overexpressing members of the Pex11 protein family in a *pex34*Δ background under conditions of peroxisome noninduction to determine whether Pex34p acts solely as a positive effector of peroxisome division, as we have observed, or if it serves a more general function in maintaining the peroxisome population even under conditions of constitutive peroxisome division.

In closing, we have shown that Pex34p is a peroxisomal protein involved in controlling peroxisome abundance under conditions of both peroxisome proliferation and constitutive peroxisome division. Pex34p controls peroxisome

numbers by itself and in cooperation with members of the Pex11 family of peroxisome divisional proteins. We also have provided insight into the roles of Pex25p and Pex34p in maintaining the population of mature peroxisomes, demonstrated that Pex27p is a late acting divisional peroxin functioning downstream of peroxisome elongation and identified Pex11p, a peroxin formerly thought to play the predominant role in peroxisome division, as an expendable divisional factor during conditions of constitutive peroxisome division (Figure 4-1). The discovery of Pex34p as a newly recognized peroxisomal protein involved in the already complex control of peroxisome population dynamics in *S. cerevisiae* emphasizes the importance that cells place on strictly regulating their peroxisome population and ensuring that they have sufficient numbers of peroxisomes to thrive under a variety of physiological and environmental conditions.

Figure 4-1: Model for peroxisome division. Under conditions of peroxisome proliferation, matrix protein synthesis is increased, resulting in an expansion of the peroxisome compartment. Pex11p, Pex25p and Pex34p act early in the peroxisome divisional process upstream of or at the stage of peroxisome elongation, while Pex27p acts later, downstream of peroxisome elongation. Under conditions of constitutive division, peroxisomes are triggered to initiate division, possibly in response to a cell cycle-regulated signal, in order to maintain peroxisome numbers during cell division. Unlike under conditions of peroxisome proliferation, Pex11p is not essential for constitutive peroxisome division, and the elongation function proposed for Pex11p may be replaced by the pulling force of the peroxisome inheritance machinery mediated by Inp2p. Together with their roles in peroxisome division, Pex25p and Pex34p may also function in maintaining the population of mature peroxisomes in the cell. Deletion of both *PEX25* and *PEX34* results in the formation of small peroxisome-like structures.


Proliferation
PEX11
PEX25
PEX34
PEX27
VPS1
DNM1
Gene Upregulation
Matrix Protein Import
PEX34
PEX25
ER
Cell
cycle
signaling?
PEX25
PEX34
INP2
PEX27
VPS1
DNM1
Constitutive
Division

CHAPTER 5

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