

Identification of Pex34p as a component of the peroxisomal de novo biogenesis machinery in yeast

Juliane Radke^a, Shirisha Nagotu^a, Wolfgang Girzalsky^a, Anirban Chakraborty^a, Markus Deckers^{a, b}, Maya Schuldiner^c, Einat Zalcvar^c, and Ralf Erdmann^{a*}

^a Abteilung für Systembiochemie, Medizinische Fakultät, Ruhr-Universität Bochum, D-44780 Bochum, Germany

^b Present address: Abteilung Biochemie II, Georg-August Universität Göttingen, D-37073 Göttingen, Germany

^cDepartment of Molecular Genetics, Weizmann Institute of Science, Rehovot 7610001, Israel.

Running Title: *Pex34p plays a role in de novo biogenesis of peroxisomes*

*Correspondence to: Ralf Erdmann, Institut für Biochemie und Pathobiochemie, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum; Tel.:49-234-322-4943; Fax.:49-234-321-4266; email.: Ralf.Erdmann@rub.de

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Summary

Cells can regulate the abundance and composition of peroxisomes to adapt to environmental changes. In the baker's yeast, *S. cerevisiae*, peroxisomes represent the only site for degradation of fatty acids. Hence, it is not surprising that growth of yeast cells on oleic acid results in a massive proliferation of peroxisomes. New peroxisomes can form either by division of pre-existing peroxisomes or de novo in a Pex25p-dependent process with the involvement of the Endoplasmic Reticulum (ER). In search for further factors involved in de novo formation of peroxisomes, we screened ~6,000 yeast mutants that were depleted of peroxisomes by conditional inhibition of *PEX19* expression. Screening the mutants for the reappearance of peroxisomes upon expression of *PEX19* identified Pex34p, in addition to the well-known component Pex25p, as crucial determinants for de novo biogenesis. Pex34p interacts with Pex19p and with different Peroxisomal Membrane Proteins (PMPs) in a *PEX19*-dependent manner. Depletion of Pex34p results in reduced numbers of import-competent peroxisomes formed de novo and Pex3p is partly retained and distributed in ER-

like structures. We suggest that Pex25p and Pex34p are both required to maintain peroxisome number in a cell and that they perform non-redundant roles in the de novo formation of peroxisomes.

Peroxisomes are widely distributed among eukaryotic organisms. They participate in a variety of metabolic processes, which are related to the metabolism of lipids and reactive oxygen species (Walter & Erdmann, 2019). The broad range of functions underlies the importance of peroxisomes for human health, as indicated by the plethora of peroxisomal disorders as well as the involvement of peroxisomes in neurodegenerative diseases, obesity, cancer, and age-related disorders (Islinger et al., 2018). Peroxisome abundance is strictly regulated by the rates of organelle formation, division and turnover (Nordgren et al., Wróblewska & van der Klei, 2019). While peroxisomes are degraded by only one main route, pexophagy (Eberhart & Kovacs, 2018, Nordgren et al.), they can be formed by two mechanisms: either by growth and division of pre-existing organelles (fission) or by de novo budding from the ER (Hettema et al., 2014).

Peroxisome fission shares some components with mitochondrial and vacuolar fission. The dynamin-like proteins Vps1p and Dnm1p as well as the Dnm1p-interacting proteins Fis1p, Mdv1p and Caf4p have all been shown to be required for peroxisome fission in *S. cerevisiae* (Hoepfner et al., 2001, Kuravi et al., 2006, Motley et al., 2008). In addition, a role for the Pex11p family of proteins in division has been established (Erdmann & Blobel, 1995, Rottensteiner et al., 2003, Tam et al., 2003). In *S. cerevisiae*, deletion or overexpression of these proteins leads to a reduced or enhanced number of peroxisomes, respectively (Rottensteiner et al., 2003, Vizeacoumar et al., 2003). A conserved role for Pex11p in peroxisome fission has been established in yeast, plants, and mammalian cells. Pex25p and Pex27p belong to the Pex11p family in *S. cerevisiae* (Rottensteiner et al., 2003, Smith et al., 2002, Tam & Rachubinski, 2002). Recent studies, however, point towards

several different roles for these proteins in peroxisome biogenesis. Pex11p was shown to be required for peroxisome membrane elongation in *Hansenula polymorpha* (Opalinski et al., 2011) and also for re-organization of peroxisome membrane proteins (Cepińska et al., 2011). However, Pex25p plays a role in the *de novo* formation of peroxisomes as shown in *H. polymorpha* (Saraya et al., 2011) and *S. cerevisiae* (Huber et al., 2012).

Pex34p has been identified as an interaction-partner of the Pex11p-family proteins and is required to maintain peroxisome number in *S. cerevisiae* (Tower et al., 2011). Moreover, Pex34p was also identified as a contact site tether between peroxisomes and mitochondria (Shai et al., 2018). Along this line, overexpression of Pex34p suppresses impaired acetate utilization in yeast lacking the mitochondrial aspartate/glutamate carrier Agc1p (Chalermwat et al., 2019). The formation of peroxisome-mitochondria contact sites from *PEX34* overexpression may facilitate function of an alternative redox shuttle through direct movement of citrate from peroxisomes to mitochondria (Chalermwat et al., 2019).

A major contribution to our understanding of peroxisome biogenesis was the genetic identification of the thirty-seven known peroxisome biogenesis factors (peroxins) (Erdmann & Kunau, 1992). Two central peroxisome biogenesis factors in yeast are Pex3p and Pex19p that together with Pex16p in higher eukaryotes seem to be directly involved in the biogenesis of the peroxisomal membrane (Jansen & van der Klei, 2019, Schrader & Pellegrini, 2017). Accordingly, mutants in Pex3p, Pex19p or Pex16p lack typical morphologically detectable peroxisomal structures (Islinger et al., 2018, Yuan et al., 2016). Peroxisomes reappear after genetic complementation as demonstrated for mammalian cells with a defect in PEX3, PEX16 or PEX19 or yeast mutants of *PEX3* and *PEX19*, indicating that they play an important role not only for peroxisomal targeting of membrane proteins but also for *de novo* formation of peroxisomes (Hettema & Motley, 2009). Pex19p, originally identified as a prenylated protein (PxF) (James et al., 1994, Kammerer et al.,

1997) or a housekeeping gene product (HK33) (Braun et al., 1994), displays a high binding affinity for a variety of peroxisomal membrane proteins (PMP) (Jansen & van der Klei, 2019). This gave rise to the idea that Pex19p acts as an import receptor and/or chaperone that binds PMPs in the cytosol, prevents their aggregation and degradation and delivers them to the peroxisomal membrane where it docks to Pex3p (Fang et al., 2004, Hettema et al., 2000, Jones et al., 2004, Shibata et al., 2004). Moreover, Pex19p-function is linked to an early step in peroxisome biogenesis namely the transfer of Pex3p from the ER to peroxisomes (Chen et al., 2014, Matsuzono & Fujiki, 2006). It is likely that additional proteins are involved in this process but have not yet been identified.

Here, we aimed to identify components required for peroxisome *de novo* biogenesis. We performed a genome-wide screen of mutants that delayed the appearance of peroxisomes after being depleted of peroxisomes and then allowed to reform them. We found that in addition to the known biogenesis factor Pex25p and other novel components also Pex34p came up as a crucial determinant. Our data show that Pex25p and Pex34p perform multiple functions in peroxisome biogenesis and maintenance, including non-redundant roles in the *de novo* formation of peroxisomes.

Materials and Methods

Strains and growth conditions - The *S. cerevisiae* strain UTL-7A (*MATa ura3-52, trp1, leu2-3/112*) was used as wild-type strain for the generation of several isogenic deletion strains as described previously (Güldener et al., 1996). Yeast strains used are listed in Table 1. Yeast complete (YPD) and minimal media (SD) used have been described previously (Erdmann et al., 1989). YNO medium contained 0.1% oleic acid, 0.05% Tween 40, 0.1% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. When necessary, auxotrophic requirements were added according to (Ausubel et al., 1992). SD medium with 0.5% galactose instead of glucose was

used for induction experiments. The screening procedure was carried out with SD-medium containing 2% galactose.

PEX34-deletion was constructed by homologous recombination based exchange of the open reading frame with KanMX4 (Güldener et al., 1996). LoxP-flanked Kan-MX4 cassettes were obtained by PCR from pUG6. Geneticin resistant colonies were selected and the loxP-KanMX4-loxP was removed by the expression of the Cre-recombinase. Double deletions *pex25Δpex34Δ* and *pex3Δpex34Δ* were made by deleting the respective gene in a *pex34Δ* strain by the exchange of the open reading frame by KanMX4. Primers RE575/862, KU365/KU700 and RE2211/22 were used respectively for *PEX3*, *PEX19* and *PEX25* deletion cassettes.

The query strain for the screening was generated as follows. First, mCherry-SKL under control of the alcohol-dehydrogenase promotor ADH1 was amplified by PCR from plasmid pAG25 using primers RE4567/RE4568. The obtained PCR product was introduced into the URA3-locus of wild-type YMS721 and isogenic *pex19Δ* strain by homologous recombination. The generated YMS721 mCherry-SKL strain was further used to integrate the *GALI*-promotor in front of the *PEX19* open reading frame. To this end, the URA-marker as well as the *GALI*-promotor was amplified by PCR from plasmid pGal-PEX3-GFP with primers RE5292/RE5293. The obtained PCR product was introduced between the native *PEX19*-promotor and the *PEX19* open reading frame by homologous recombination.

Generation of a screening library-The generated query strain was crossed into the yeast deletion, hypomorphic allele and mini-peroxi collections (Breslow et al., 2008, Gabay-Maskit et al., 2020, Giaever et al., 2002) by the synthetic genetic array method (Cohen & Schuldiner, 2011, Tong & Boone, 2006). This procedure resulted in a collection of haploid strains used for screening mutants that display peroxisomal abnormalities after re-expression of Pex19p.

Automated high-throughput fluorescence microscopy- The screening collection, containing a total of 6321 strains, were grown for several generations on solid glucose medium to deplete peroxisomes, then for 12 h in 2% galactose medium at 30°C under gently shaking. 5µl of each culture were shifted to 95µl fresh galactose-medium. After additional 4 h growth, cells were visualized during mid-logarithmic growth using an automated microscopy setup as described previously (Breker et al., 2013).

Plasmid constructions- Plasmids and oligonucleotides used are listed in Table II and III. Plasmid GFP-Pex34 was constructed using the plasmid pUG36 as a template and primers RE1524, RE1582 for PCR. Plasmid pIH024 expressing Pex3-GFP fusion under the control of the Gal4-promotor was constructed as follows: The coding region of Pex3-GFP was amplified by PCR with primers RE1273 and RE1274 and plasmid pIH643 (Heiland & Erdmann, 2005) as template. The PCR product was digested with XhoI/BamHI and cloned into XhoI/BamHI sites of pGal4 (Clontech). For C-terminal GFP-fusion of Pex25, the coding region of Pex25 was amplified from the genome by PCR with primers Pex25-GFP-fw/Pex25-GFP-rev and introduced into EcoRI/SalI sites of pUG35. All constructs were confirmed by DNA sequencing. The GFP-SKL and DsRed-SKL plasmids have been described previously (Brocard et al., 1997, Halbach et al., 2009).

Two-hybrid Analysis- The two hybrid analysis was based on the method of (Fields & Sternglanz, 1994). The tested genes were fused to the DNA-binding domain (Gal-DB) or trans-activating domain (Gal-AD) of Gal4 in the vectors pPC86 and pPC97 (Chevray & Nathans, 1992). Co-transformation of two hybrid vectors into strain PCY2 was performed according to (Gietz & Woods, 1994). Transformed yeast cells were plated on SD synthetic medium without tryptophan and leucine. β -galactosidase filter assays were performed as described earlier (Rehling et al., 1996b).

Image acquisition- Fluorescence microscopy images were recorded on an AxioPlan 2 microscope (Zeiss, Jena) equipped with a α Plan-FLUAR 100 \times /1.45 oil objective and an AxioCam MRm camera (Zeiss, Jena) at room temperature. If necessary, contrast was linearly adjusted using the image acquisition software AxioVision 4.8 (Zeiss, Jena).

Miscellaneous- Immunoblotting was performed with polyclonal rabbit antibodies raised against Pex19p (Götte et al., 1998) and mitochondrial porin (Kerssen et al., 2006).

Results

Construction of a conditional Pex19p-mutant strain

Cells deficient in Pex19p are characterized by the lack of peroxisomes, with the consequence that the peroxisomal matrix proteins are mislocalized to the cytosol. New peroxisomes can form *de novo* in these cells, upon reintroduction of *PEX19* (Götte et al., 1998). To carry out a genome wide screen to identify components involved in this process, we first generated a query strain that enables us to control and monitor *de novo* formation of peroxisomes. To this end, the *GAL4*-promotor was integrated in front of the *PEX19*-coding region, replacing the endogenous *PEX19*-promotor (**FIG1A**) in a yeast strain compatible with automated mating, sporulation and segregant selection. This procedure enabled us to switch off *PEX19*-expression on glucose medium, whereas presence of galactose induced *PEX19* expression. To monitor formation of functional and thus import-competent peroxisomes, the fluorophore mCherry fused to the peroxisomal targeting-signal type 1 (PTS1: SKL) under control of the constitutive *ADH1*-promotor was additionally integrated into the genome of the query strain (**FIG1A**).

To analyze the regulation of *PEX19*-expression, cells of the control, *pex19 Δ* strain and the newly generated query strain (*Gal4-PEX19*) were first grown on glucose-medium, subsequently shifted to either glucose or galactose containing medium and whole cell lysates thereof were analyzed for

Pex19p-abundance. Mitochondrial porin served as an internal loading control and did not differ between the analyzed samples. In control cells, the Pex19p-steady-state level was nearly the same under both, glucose- and galactose-conditions, whereas no Pex19p-positive signal was obtained in samples of the *pex19Δ* mutant as expected (**FIG1B**). In the query strain, Pex19p was not detected in the lysates from glucose-grown cells, indicative of a tight repression. In contrast, the lysates from galactose-induced cells contained similar amounts of Pex19p as wild-type cells. (**FIG1B**). This clearly demonstrated a tight glucose-repression and efficient galactose-induction of *PEX19* in the query strain.

Although the Pex19p-concentration in our query strain was below the detection level when glucose served as a carbon source, we confirmed that the basal expression is low enough to block peroxisome biogenesis and that growth on glucose-medium for several generations sufficiently depleted peroxisomes of the parental strain. To this end, the query strain was compared with wild-type and *pex19Δ* cells for the presence of import-competent peroxisomes in glucose-grown and galactose-induced cells, monitored by fluorescence microscopy. In line with a peroxisomal localization, a punctuate pattern of the genomically expressed mCherry-SKL was seen in control cells, independent of the growth conditions (**FIG1C**). As expected for a strain impaired in peroxisome biogenesis (Distel et al., 1996), the synthetic peroxisomal marker was mislocalized to the cytosol and thus caused a diffuse staining in the *pex19Δ* strain. While under glucose conditions the query strain behaved like a *pex19Δ* mutant, red puncta were monitored when *PEX19*-expression was induced by the presence of galactose (**FIG1C**). We quantified that no more than 6 % of the query cells maintained import-competent peroxisomes even under glucose conditions (and those that did had no more than a single one) making this strain highly suitable to screen for components of the peroxisomal *de novo* biogenesis.

Screening for components required for the peroxisomal de-novo biogenesis.

The created query strain was compatible with the Synthetic Genetic Array (SGA) automated mating approach in yeast (Cohen & Schuldiner, 2011, Tong & Boone, 2006). To this end, this strain was crossed into the nearly 5000 strains of the deletion library (Giaever et al., 2002) and more than 1000 strains of the DAmP (Decreased Abundance by mRNA Perturbation) hypomorphic allele library for essential genes. Following sporulation and selection of haploids, about ~6000 unique haploid strains were obtained, each with a deletion of a single yeast gene and harboring *GAL4-PEX19* as well as mCherry-SKL (**FIG 2**). To perform the screen, the generated screening library was first grown on glucose medium with intermediate medium changes to force peroxisome depletion. Thereafter, cells were shifted to galactose medium and grown for 16 h again with intermediate medium change to induce Pex19p-expression. Finally, cells were subjected to microscopic inspection and automated image acquisition. Using an automated high-content microscopy setup specifically configured for this purpose (Breker et al., 2013), three images of one focal plane were recorded for each mutant covering hundreds of cells per strain. Images were analyzed for the appearance of fluorescence labelling differing from that of control cells. Analyzed strains were grouped into those with no obvious difference to the control and those that contain mature peroxisomes that differed from control cells by number or size. Finally, we found strains that had complete mislocalization of the peroxisomal marker mCherry-SKL to the cytosol or a mixed phenotype (**FIG2**).

In total, 100 strains were identified, which differed from the control in peroxisome appearance (**Fig 2B; Supplementary table 1**) and were defined as hits. Eighteen of them turned out to be affected in one of the known PEX-genes, which encode peroxisomal assembly factors (Islinger et al., 2010). In two strains (*YGL152C*, *YJL211C*), the corresponding gene is considered to be dubious but is overlapping a PEX gene: *PEX14* and *PEX2* respectively, and the deletion would have deleted

them as well. It had been demonstrated previously that yeast mutants lacking peroxisomes require the presence of Pex3p and Pex25p to regenerate this organelle *de novo* (Huber et al., 2012, Saleem et al., 2008). Accordingly, *pex3Δ* and *pex25Δ* both displayed eye-catching phenotypes. The *pex3Δ* mutant cells displayed a distinct cytosolic labelling of mCherry-SKL, which is typical for a strain affected in peroxisomal matrix protein import or biogenesis (**FIG 3**; (Distel et al., 1996)). The *pex25Δ* cells showed less peroxisomes and a partial mislocalization of the peroxisomal marker to the cytosol (**FIG 3**). The fact that we identified Pex3p and Pex25p, two known components of the *de novo* biogenesis (Huber et al., 2012, Saleem et al., 2008), and the finding that the hits of our screen cover almost all components known to be required for import of type1 peroxisomal matrix-proteins or peroxisomal appearance, demonstrated the validity of the screen.

However, there were many additional hits that were previously not identified as taking part in this process but have strong connections to peroxisomes and may be important to further study. For example: A deletion of *SADI* caused the appearance of multiple but smaller peroxisomes. Sad1p is involved in pre-mRNA-splicing (Lygerou et al., 1999). In this respect, it is interesting to note that several of the genes identified in the screen contain introns (*NHP6B*, *PCH2*, *RPL14A*, *RPL37A* and *RPL40A*). A deletion of *DOA4* caused a mixed phenotype of normal import and a partial import defect (**FIG 3**). Doa4p was shown to be involved in cleavage of ubiquitin chains and is required for turnover of the PTS2 co-receptor Pex18p, which also is ubiquitinated (Papa & Hochstrasser, 1993, Purdue & Lazarow, 2001). Growth-rate of *doa4Δ* cells was shown to be reduced on both, glucose and oleic-acid conditions, and matrix protein import is affected (Debelyy et al., 2011).

An additional interesting hit is Mdh2p whose deletion caused a mixed phenotype mixed phenotype of normal import and a partial import defect. Mdh2 was described as an enzyme involved in both, the glyoxylate cycle and fatty acid β -oxidation (Kunze et al., 2006). Initially described as a cytosolic protein, recent data demonstrated a fraction of Mdh2p to be localized to peroxisome,

depending on Pex5p as well as Mdh3p (Gabay-Maskit et al., 2020). Deletion of *YAL016C-B* - a dubious open reading frame that overlaps with the promotor region of *TPD3* caused a mixed phenotype similar to those observed in *doa4Δ* and *mdh2Δ* cells. Tpd3p represents a subunit of the PP2A complex, which is a subgroup of the PPP-(serine/threonine-specific phosphoprotein phosphatases) family. These phosphatases are conserved among eukaryotes and a peroxisomal localization was reported for the protein from *Arabidopsis thaliana* (Kataya et al., 2019, Lillo et al., 2014). Recently, regulation of peroxisomal matrix protein import via phosphorylation of Pex14p, a key component of the import machinery, was reported, it may be that yeast Tpd3p is involved in this process (Schummer et al., 2020). Shr5p (Erf4) is involved in the palmitoylation and subcellular localization of yeast Ras protein (Zhao et al., 2002). *shr5Δ* appeared in our screen with a mixed phenotype. As Pex19p itself is farnesylated, the observed phenotype might be due to less or lacking Pex19p-modification. We also identified *vps1Δ* by a reduced peroxisome number, a phenotype, which was also reported previously (Hoepfner et al., 2001, Kuravi et al., 2006). Vps1p is required for fission of existing peroxisomes and acts together with Mdm1p (Motley & Hettema, 2007). An additional interesting hit of our screen with a mixed phenotype was *VPS13*. In mammals, the VPS13 gene family consists of VPS13A-D. Recently, VPS13D was shown to be involved in regulation of peroxisomal biogenesis and bridging of the ER to mitochondria and peroxisomes (Baldwin et al., 2021, Guillén-Samander et al., 2021). Deletion of *sym1Δ* gave a dramatic phenotype of reduced number of peroxisomes (**FIG 3**). Sym1p belongs to the PXMP2 protein-family, named by human PXMP2, a channel forming protein of mammalian peroxisomes (Rokka et al., 2009). Recently, Pex37p of *Hansenula polymorpha* was identified, which also belongs to this protein-family. A deletion of *PEX37* displays, like *sym1Δ*, a reduced number of peroxisomes (Singh et al., 2019), indicating that Sym1p might be the functional orthologue of Pex37p in *S. cerevisiae*.

For a more detailed analysis, we chose to focus on one of the most severe hits, the gene *PEX34*. Pex34p was originally reported to function in concert with the Pex11p-protein family members Pex11p, Pex25p, and Pex27p to control the peroxisome populations of cells under conditions of both peroxisome proliferation and constitutive peroxisome division (Rottensteiner et al., 2003, Tam et al., 2003, Tower et al., 2011). However, Pex25p also plays a role in the de novo synthesis of peroxisomes (Huber et al., 2012). As Pex34p has been reported to interact with Pex25p (Huber et al., 2012), we considered that both proteins might function together in peroxisomal de novo synthesis.

Interaction of Pex34p-Pex19p

Pex34p was originally identified as a peroxisomal membrane protein, which are typically bound by the import receptor Pex19p prior to peroxisomal targeting (Rottensteiner et al., 2004, Tower et al., 2011). The targeting signal for peroxisomal membrane proteins is defined as a Pex19p-binding motif in conjunction with a transmembrane segment or a peroxisomal anchor sequence (Girzalsky et al., 2006, Rottensteiner et al., 2004). The use of topology prediction programs revealed that Pex34p contains two transmembrane segments ((Tower et al., 2011), **FIG 4A**). Moreover, we identified two regions (amino acids 58-72 and 133-143) fitting to the consensus sequence of putative Pex19p-binding sites, which are located in close proximity to the *in silico* predicted transmembrane spanning regions (**FIG4A**). Thus, Pex34p seems to be a typical Pex19p-cargo. To support this assumption, we investigated whether Pex19p directly interacts with Pex34p by means of a yeast two-hybrid system. Gal4p-fusions of activation domain (AD)-Pex34p and DNA-binding domain (DB) of Pex19p were co-expressed in the *S. cerevisiae* Y2H-host strain PCY2 and interactions were monitored by β -galactosidase filter assays. In line with the idea of Pex34p being a cargo of Pex19p, a clear interaction was indicated by our Y2H-result (**FIG4B**).

Pex34p is required for proliferation of newly formed peroxisomes in pex3Δpex34Δ cells-

Considering a function of Pex34p in peroxisomal *de novo* synthesis, we had a closer look in re-appearance of peroxisomes in selected single and double mutant strains. To this end, we applied an established assay for *de novo* synthesis based on a plasmid-encoded *PEX3* under regulation of the *GAL4*-promotor (Hoepfner et al., 2005). Similarly to Pex19p, the role of Pex3p in *de novo* biogenesis of peroxisomes has been established (Aranovich et al., 2014, Ma et al., 2011, van der Zand et al., 2010). This assay also allowed the direct monitoring of the expression and localization of GFP-tagged Pex3p. To this end, a *pex34Δpex3Δ* double deletion was constructed that lacks peroxisomal structures (data not shown). For the *de novo* restoration of peroxisomes in these cells, Pex3p-GFP fusion protein, expressed under the control of the GAL promoter, was reintroduced into the cells. To this end, cells were first pre-cultured on glucose medium, which represses the GAL promoter. Cells were then shifted to medium containing 0.5% galactose for the induction of Pex3p-GFP and initiation of *de novo* peroxisome formation, which was monitored by fluorescence microscopy. The *pex3Δ* cells with GAL-induced Pex3p-GFP served as control. At T0 (just after shifting to galactose containing medium), a detectable Pex3p-GFP signal was not observed in the cells (**FIG 5A**). After 2hrs of induction, Pex3p-GFP puncta were visible, which increased in size and number with time in the *pex3Δ* control cells (**FIG5A**). In contrast, peroxisome number was significantly reduced in *pex34Δpex3Δ* cells upon reintroduction of Pex3p-GFP (**FIG5A, B**). Pex3p-GFP was observed as a single spot after two hours of induction and spot number did not significantly increase with induction-time (**FIG5A, B**). The single punctate structure that contained Pex3p-GFP was confirmed to be an import competent peroxisome as shown by co-labelling with DsRed-SKL (**FIG 5C**). Important to note, we also observed a fraction of Pex3-GFP in a reticulated

membrane structure, presumably the ER (**FIG 5A**). We take this finding as indication of a partial targeting to or longer retention of Pex3p in the ER.

Reintroduction of Pex34 in $pex25\Delta pex34\Delta$ cells does not fully restore peroxisome formation-

Upon induction, mutants lacking either Pex25p or Pex34p are characterized by a reduced number of import-competent peroxisomes (Tower et al., 2011, Vizeacoumar et al., 2003). In contrast, the phenotype of the corresponding double mutant is more severe with most cells being characterized by a greater reduction in peroxisome number when compared to the single deletion strains and a mislocalization of at least one peroxisomal matrix protein to the cytosol (Tower et al., 2011). Moreover, Pex25p has been reported to be required for the *de novo*-formation of peroxisomes (Huber et al., 2012, Saraya et al., 2011). To gain more insight into a putative concerted function of Pex34p and Pex25p, we first compared the phenotype of $pex34\Delta pex25\Delta$ cells upon inducing and non-inducing conditions. Upon induction of peroxisome proliferation by growth on oleic-acid medium, a mixed phenotype was observed. As judged by the localization of the peroxisomal marker GFP-SKL, this marker is mislocalized to the cytosol in a population of oleic acid-induced $pex34\Delta pex25\Delta$ cells, indicating that the double mutant exhibits an import defect for peroxisomal matrix proteins (**FIG 6A**). However, this phenotype is not evident on glucose medium, where most of the cells have labeled peroxisomes (**FIG 6A**). The less pronounced phenotype of the $pex34\Delta pex25\Delta$ mutant grown under non-inducing conditions might indicate that a low rate of peroxisome formation can still take place normally in the absence Pex34p and Pex25p. On the other hand, the import defect that is seen under inducing conditions might indicate that a higher rate of peroxisome formation requires the presence of both proteins.

As the phenotypes of the single and double mutants differ significantly, we analyzed their complementation with either of the two proteins. To this end, we reintroduced GFP-Pex34p into

pex34Δpex25Δ strain to investigate whether the double mutant cells that are obviously devoid of peroxisomes form functional peroxisomes upon complementation of one of the defects. Surprisingly, we again observed a mixed phenotype. In cells containing punctuate mCherry-SKL, co-localization of GFP-Pex34p was observed indicating a peroxisomal localization of the protein (**FIG 6B**). However, in cells with no detectable peroxisomal structures (cytosolic mCherry-SKL), GFP-Pex34p was observed to be localized to single spots. This result indicates that complementation of *pex34Δpex25Δ* cells with Pex34p does not fully rescue the phenotype. Similarly, we also reintroduced Pex25p-GFP in *pex34Δpex25Δ* cells and analyzed whether the mutant phenotype of these cells could be rescued by Pex25p. However, a similar localization pattern as for Pex34p was observed. Thus, single deletions of Pex25p or Pex34p results in a reduced number of functional peroxisomes but upon the concurrent depletion of both proteins a fraction of cells is devoid of peroxisomes, a phenotype which cannot be rescued by complementation with either Pex34p or Pex25p. Accordingly, both proteins play non-redundant roles in the *de novo* formation of peroxisomes.

Discussion

Peroxisomes are formed by either growth and division of existing organelles or *de novo* with the involvement of the ER and the peroxisomal membrane protein Pex3p (Haan et al., 2006, Hoepfner et al., 2005, Kragt et al., 2005, Titorenko et al., 1997). In yeast cells, growth and division is the major source for new peroxisomes under normal conditions (Motley & Hettema, 2007), whereas under conditions in which peroxisomes are absent (e.g. segregation defect), newly synthesized Pex3p targets to the ER, concentrates at the membrane into foci and buds off as vesicles which later import matrix proteins and transform into mature peroxisomes (Hoepfner et al., 2005). Here, we performed a genome wide screen for strains affected in peroxisomal *de novo* biogenesis. A

conditional strain was generated in which Pex19p-expression was induced by presence of galactose and repressed in glucose-containing medium. Growth in glucose resulted in a complete absence of Pex19p and depletion of peroxisomes from the cells (**FIG 1**). Upon induction of *PEX19* expression, these cells were able to form new peroxisomes *de novo*. Combined with about 6000 single-gene deletions, we identified 100 strains, which display peroxisomes that differ from wild-type organelles in terms of number, size or import capacity. Among the hits, a number of strains lacking proteins known to be required for proper peroxisome biogenesis (PEX-proteins) appeared, demonstrating the validity of the screen (**FIG 2, Supplementary table 1**).

One hit of our screen was the *sym1Δ* mutant. This strain differed from the control by a reduced number of peroxisomes upon induction of peroxisomal *de novo* synthesis (**FIG 3**). Sym1p (for “stress-inducible yeast Mpv17”), is a heat-induced gene product, with a role in ethanol metabolism and tolerance and a homologue to mammalian Mpv17 (Antonenkova et al., 2015, Trott & Morano, 2004). Both, Sym1p and Mpv17p belong to the PXMP2-family, which additionally includes Pxmp2p, Mpv17-like protein (M-LP), and Fksg24p (Mpv17-like 2 protein)) in mammals and Yor292p in yeast (Antonenkova et al., 2015). In contrast to the peroxisomal localization of Pxmp2p, the Mpv17p, MP-L, and Mpv17L2p proteins are mitochondrial (Rokka et al., 2009). The same is true for Sym1p, which is an integral membrane protein of the inner mitochondrial membrane (Trott & Morano, 2004). Recently, Pex37p was identified as an additional family member in the methylotrophic yeast *Hansenula polymorpha* (Singh et al., 2019). The corresponding *pex37Δ* strain was not affected in peroxisome biogenesis or proliferation under peroxisome inducing condition. However, loss of Pex37p caused a significant reduction in peroxisome number under peroxisome repressing conditions, like growth on glucose (Singh et al., 2019). The fact that also peroxisome segregation was affected in *pex37Δ* led to the suggestion that in addition to Pex11p and Dnm1p, Pex37p plays a role in peroxisome fission under glucose condition. Except for the fact that Sym1p

was shown to be a mitochondrial protein, it plays a role for peroxisomal abundance similar to that of Pex37p. It is tempting to speculate that based on the similar phenotype and the affiliation to the same protein family that Sym1p is the Pex37p homologue in *S. cerevisiae*. However, additional studies are required, to support this assumption.

We chose to focus on the *pex34Δ* strain that displayed a reduced number of peroxisomes upon *de novo* synthesis conditions (**Supplementary table 1, FIG 3**). Originally, Pex34p was identified by a large-scale protein interaction screen that identified the protein as a binding partner of a number of peroxisomal biogenesis factors (Yu et al (2008)). Later on, it was discovered that Pex34p functions together with Pex11p family members in peroxisome biogenesis (Tower et al., 2011), originally based on the 2-hybrid interactions between Pex34p and the Pex11p family members (Pex11p, Pex25p and Pex27p). More recently, Pex34p was characterized as a tether protein involved in peroxisome-mitochondria (PerMit) contacts and it was shown to contribute to β -oxidation of fatty acids (Shai et al., 2018). Our data suggest that Pex34p also performs a role in the *de novo* synthesis of peroxisomes, which may or may not be due to its role in mediating contact with mitochondria. In this respect, it is interesting to note that members of the Pex11p-family that interact with Pex34p are also involved in the *de novo* formation of peroxisomes (Opalinski et al., 2011). Our results support the notion of a requirement of Pex34p for an efficient exit of Pex3p from the ER. Accordingly, reintroduction of Pex3p-GFP in *pex34Δpex3Δ* cells did not completely restore the formation of peroxisomes (**FIG 5**). When expressed in *pex3Δ* cells, the Pex3p-fusion protein completely localized to peroxisomes, which form *de novo* and proliferate normally under inducing conditions so that the complemented cells are characterized by at least a few peroxisomes. In contrast, when expressed in *pex3Δpex34Δ* cells, the Pex3p-GFP at least partly retains in structures, which might be the ER, and its expression leads to the formation of mostly one

peroxisome per cell. This finding might be explained by different possibilities namely 1) defect in the fission of peroxisomes 2) defect in the *de novo* formation of peroxisomes. However, a sole defect in fission of peroxisomes might be difficult to reconcile with the possible ER-retention of the membrane marker Pex3p, unless the defect might feedback and slow down the *de novo* synthesis. With respect to a possible function for Pex34p in the formation of peroxisomes from the ER, it is tempting to speculate that the observed phenotype of mostly one peroxisome per cell might be explained by an inefficient budding of newly formed peroxisomes from the ER. The budding defect is accompanied by the observed retention of Pex3p at the ER in the absence Pex34p, which might be the cause or the consequence of the defect. The fact that Pex3p under these circumstances is distributed throughout the ER might be explained by a role for Pex34p in Pex3p recruitment to the budding zone.

As Pex25p has also been implicated in *de novo* synthesis of peroxisomes in *H. polymorpha* (Saraya et al., 2011) and *S. cerevisiae* (Huber et al., 2012), and as Pex25p and Pex34p do interact, we investigated whether both proteins function in concert in the *de novo* formation of peroxisomes and studied this process in the *pex34Δpex25Δ* double mutant. Cells concurrently lacking Pex25p and Pex34p are characterized by a severe import defect for the otherwise peroxisomal Mdh2-GFP (Tower et al., 2011) and GFP-SKL under inducing conditions (**FIG 6**). Remarkably, the peroxisome biogenesis defect of the double mutant was not effectively rescued by complementation with either Pex34p or Pex25p reinforcing the non-redundant function of these proteins (**FIG 6**).

The data presented show that Pex34p and Pex25p function in concert in the maintenance of peroxisome abundance and in the *de novo* synthesis of peroxisomes from the ER. The detailed role of Pex34p functions in these two processes is still unknown. The distribution of Pex3p in the ER upon Pex34p-depletion would be in agreement with the role of a Pex3p-specific ER-recruitment

factor. The fact that Pex19p can bridge the interaction of Pex34p with other PMPs is unusual and might be explained by a non-typical Pex19p-Pex34p interaction, which needs to be further investigated.

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Table I.S. *cerevisiae* strains used in this study

Strain	Description	Source or Reference
UTL-7A	<i>Mata, ura3-52, trp1, leu2-3 112</i>	(Erdmann et al., 1989)
<i>pex34Δ</i>	<i>Mata, ura3-52, trp1, leu2-3 112, pex34Δ::loxP</i>	This study
<i>pex19Δ</i>	<i>Mata, ura3-52, trp1, leu2-3 112, pex19Δ::loxP</i>	(Halbach et al., 2009)
<i>pex14Δ</i>	<i>Mata, ura3-52, trp1, leu2-3 112, pex14Δ::loxP</i>	(Girzalsky et al., 1999)
<i>pex34Δ pex3Δ</i>	<i>Mata, ura3-52, trp1, leu2-3 112, pex34Δ::loxP, pex3Δ::loxP</i>	This study
<i>pex34Δ pex25Δ</i>	<i>Mata, ura3-52, trp1, leu2-3 112, pex34Δ::loxP, pex25Δ::loxP</i>	This study
PCY2 (WT)	<i>Mata, Δgal4, Δgal80, URA3::GAL1-lacZ, lys2-801^{amber}, his3-Δ200, trp1-Δ63, leu2 ade2-101^{ochre}</i>	(Chevray & Nathans, 1992)
YMS721	<i>S288C; MATα; his3Δ1, leu2Δ0, lys2+/lys+, met15Δ0 ura3Δ0, can1Δ::STE2pr-sp, HIS5, lyp1Δ::STE3pr-LEU2</i>	M. Schuldiner
YMS721 mCherry-SKL GALp-PEX19	<i>S288C; MATα; his3Δ 1, leu2Δ0, lys2+/lys+, met15Δ0, ura3Δ::PADH1-mCherry-SKL-TADH1-PTEF-nat1-TTEF, can1Δ::STE2pr-sp, HIS5, lyp1Δ::STE3pr-LEU2, pex19::URA3-PGAL1</i>	This study
YMS721 mCherry-SKL, <i>pex19Δ::URA3</i>	<i>S288C; MATα; his3Δ 1, leu2Δ 0, lys2+/lys+, met15Δ 0, ura3Δ::PADH1-mCherry-SKL-TADH1-PTEF-nat1-TTEF, can1Δ::STE2pr-sp, HIS5, lyp1Δ::STE3pr-LEU2, pex19::URA3-PGAL1, PEX3-eGFP</i>	

Table II Plasmids used in this study

Plasmid	Description	Source / Reference	Primers
pPC86-	Gal4p(DB)	(Chevray & Nathans, 1992)	
pPC97-	Gal4p(AD)	(Chevray & Nathans, 1992)	
pPC86-PEX19	Gal4p(DB)-Pex19p	(Rottensteiner et al., 2004)	
pPC86-PEX34	Gal4p(DB)-Pex34p	This study	RE1524/RE1525
pUG6	Deletion, Cre-Recombinase	(Güldener et al., 1996)	
pUG36	GFP	(Niedenthal et al., 1996)	
pUG36-PEX34	GFP-Pex34p	This study	RE1524/RE1582

pUG35-PEX25	Pex25p-GFP	This study	Pex25p-GFP- fw/Pex25p-GFP- rev
pRS414-Ds-Red-SKL	DsRed-SKL (-trp)	(Halbach et al., 2009)	RE1685/RE1686
pGal-PEX3-GFP	Pex3p-GFP	This study	RE1273/RE1274

Table III Oligonucleotides used in this study

Primer	Sequence 5' -> 3'
RE575	CGTAAAAGCAGAAGCACGAAACAAGGAGGCCAAACCACTAAAAGGCGTACGCTG CAGGTCGAC
RE862	CATGTTACCTATTGCACACTTACTGTATTAAGATTACGCATAGGCCACTAGTGG ATCTG
RE1273	CTCGAGATGGCCCCAAATCAAAGA
RE1274	GGATCCTTATTTGTACAATTCATCCATACC
RE1524	AGGTCGACCCCGGGAATGGTTTCGAAGAAAAATACGG
RE1525	TAGCGGCCGCATTATACAATTATTCTACAAAGTGTT
RE1582	GGCTCGAGTTATACAATTATTCTACAAAGTGTT
Pex25p-GFP- fw	GATCGAATTCATGAGTCAGTTTGGCACGAC
Pex25p-GFP- rev	GATCGTCGACATCTTTTGAAGAGCAAAGTG
RE3236	AAAGAGGAGATGAATAAAAGTAAAGGAAGAAGAAAAGAAAGTTGAGAACAAAA GCTGGAGCTCAT
RE3237	CCATATATCTTTTGCACCTGATTTTCAGCCGTATTTTCTTCGAAACCTTATCGTCATC ATCAAGTG
KU365	AAGAATTACAAATTGTGGGAACCGAAGTATTGACGGAAAGAAGAAATGCGTACG CTGCAGGTCGAC
KU700	TACTTTTTTTTTTTTTTTTTTTTACTGTTATCATAAATATATATACCTTAATAGGCCAC TAGTGGATCTG
RE4567	GTGAAGGATAAGTTTGACCATCAAAGAAGGTTAATGTGGCTGTGGTTTCGCTGCA GGTCGACGGATCGA
RE4568	AAAGGCCATGAAGCTTTTTCTTTCCAATTTTTTTTTTTTCGTCATTATAGTCGACAC TGGATGGCGGCGT
RE5292	AAGTATTGACGGAAAGAAGAAATGCCAAACATACAACACGAAGTAGTGCACCAT ACCACAG
RE5293	AAGGTCATCCAAATCATCAAAATTATCGTACTCGTTTTTCATTCATATCGATGGTTT TTTCTCTTG

Figure legends

Figure 1: Generation and characterization of the query strain for a genome-wide screen.

(A) Schematic representation of the genetic modification of strain YMS721. The GAL4-promotor was introduced by homologous recombination upstream of the *PEX19*-coding region. mCherry fused to PTS1-signal SKL under control of the ADH1-promotor was integrated into the *URA3*-locus. (B) Strains as indicated were first grown to mid log-phase on glucose-medium and then

shifted to either glucose (Glc) or galactose (Gal) conditions. After growth for 4 h, whole cell TCA-lysates were prepared. Equal portions were subjected to SDS-PAGE followed by immunoblot analysis using anti-Pex19p and anti-porin (mitochondrial marker protein, served as loading control) antibodies. (C) Indicated cells were grown in glucose medium until mid-log-phase, shifted after a washing step to galactose conditions and grown for 4 h. Prior and after growth on galactose, cells were analyzed by fluorescence microscopy for localization of mCherry-SKL. The scale bar represents 5 μ m.

FIGURE 2: A Genome-wide screen for components involved in peroxisomal de-novo biogenesis

(A) Schematic workflow of the genome-wide screen. *GAL4-PEX19/mCherry-SKL* were integrated into yeast strain YMS721 to generate the query strain. This was crossed with the indicated deletion/DAmP libraries, sporulated and selected for haploid deletion-strains harboring both the *GAL4-PEX19/mCherry-SKL* as well as single gene-deletions. Strains were analyzed by automated microscopy and were grouped into indicated phenotypes based on the appearance of the peroxisomal marker mCherry-SKL. B) List of mutants that display a localization of mCherry-SKL different from that of wild-type cells. C- cytosolic distribution of the reporter; S – small peroxisomes; L – large peroxisomes; R – reduced number of peroxisomes; El – elongated peroxisomes; M – mixed phenotype

FIGURE 3: Examples of hits from the genome wide-screen

Examples of mutants from the genome-wide screen. Images of strains from the genome-wide screen containing *GAL4-PEX19* and mCherry-SKL. Strains were first grown on glucose-medium and then shifted to galactose-medium to induce Pex19p expression. Images were taken after 16h galactose-induction with intermediate medium changes. The scale bar represents 5 μ m.

Figure 4: Pex34p interacts with Pex19p

(A) Schematic representation (upper panel) of Pex34p with numbers representing position of corresponding amino-acid residues. Hypothetic transmembrane segments (TM1 and TM2) and Pex19p-binding motives (mPTS1 and mPTS2) are highlighted. Lower panel: Alignment of the consensus sequence for Pex19p-binding motif (Rottensteiner et al., 2004) with the two identified motifs (mPTS1 and mPTS2) within Pex34p. Position are indicated by numbers, the asterisk indicates the stop-codon. (B) Double transformants of the two-hybrid reporter strain PCY2 expressing the indicated GAL-fusion proteins, were selected and β -galactosidase activity was determined by a filter assay with X-Gal as substrate. Three representative independent double transformants are shown.

Figure 5: Pex34p is required for efficient re-introduction of peroxisomes

(A) Pex3p-GFP plasmid under the control of the inducible *GAL4*-promoter was transformed into *pex3 Δ* and *pex3 Δ pex34 Δ* cells. Cells were precultured on repressing conditions (glucose medium) and then shifted to medium with 0.5% galactose to induce Pex3p-GFP and monitor peroxisome formation at indicated time points by fluorescence microscopy. (B) For quantification, peroxisomes of hundred cells of each strain (depicted in A) at indicated time-points were counted for two different experiments and the average number is displayed. In *pex3 Δ* cells, after two hours of cultivation on galactose medium, Pex3p-GFP was observed in punctuate peroxisomal structures, which proliferated upon prolonged cultivation. In *pex3 Δ pex34 Δ* cells after two hours of induction, Pex3p-GFP localized to a single puncta and additionally to ER-like structures. (C) The single

Pex3p-GFP puncta present in *pex34Δpex34Δ* cells upon Pex3p-GFP expression co-localized with the peroxisomal matrix marker DsRed-SKL and thus represent import-competent peroxisomes. The scale bar in panels A and C represents 5 μm.

Figure 6: Reintroduction of GFP-Pex34p or Pex25p-GFP in *pex34Δpex25Δ* cells does not restore peroxisome formation

(A) GFP-SKL plasmid was transformed to wild-type and *pex34Δpex25Δ* cells. Peroxisome morphology and abundance of cells grown on glucose and on oleic acid was analyzed by fluorescence microscopy. Cells were precultured on glucose and were shifted to glucose or oleic acid medium. Many *pex34Δpex25Δ* cells showed mislocalization of GFP-SKL when grown on oleic acid medium. The scale bar represents 2.5 μm.

(B) Plasmid GFP-Pex34p or (C) Pex25p-GFP was transformed to *pex34Δpex25Δ* cells and GFP-localization was analyzed by fluorescence microscopy. Cells that displayed a normal peroxisomal localization of both GFP-Pex34p and Pex25p-GFP also exhibited a normal import of synthetic marker protein mCherry-SKL, indicated by the clear co-localization. In contrast, when Pex25p or Pex34p appeared to be mislocalized, the peroxisomal matrix protein import was affected and the marker proteins accumulated in the cytosol. The scale bar represents 5 μm.

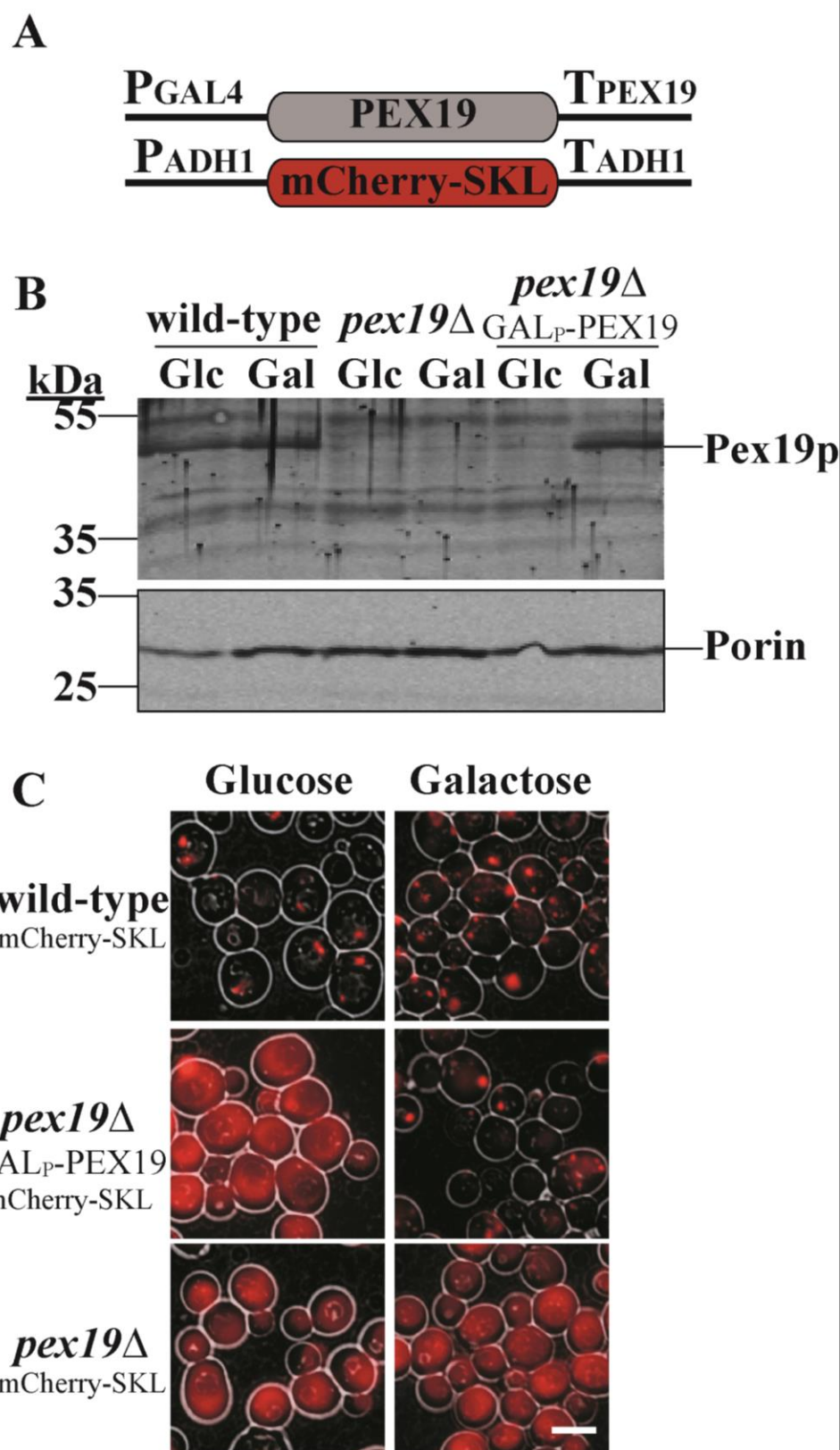


FIGURE 1

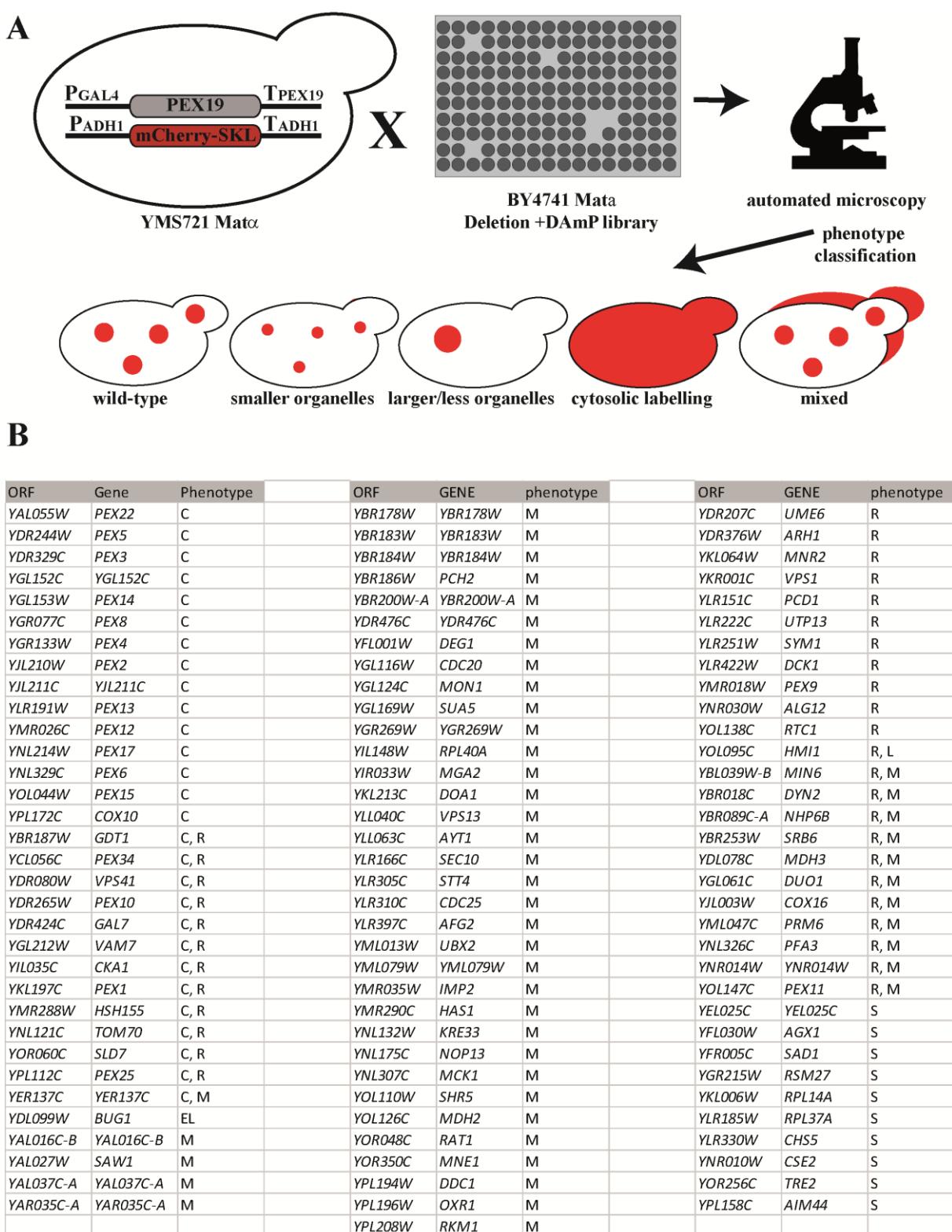


FIGURE 2

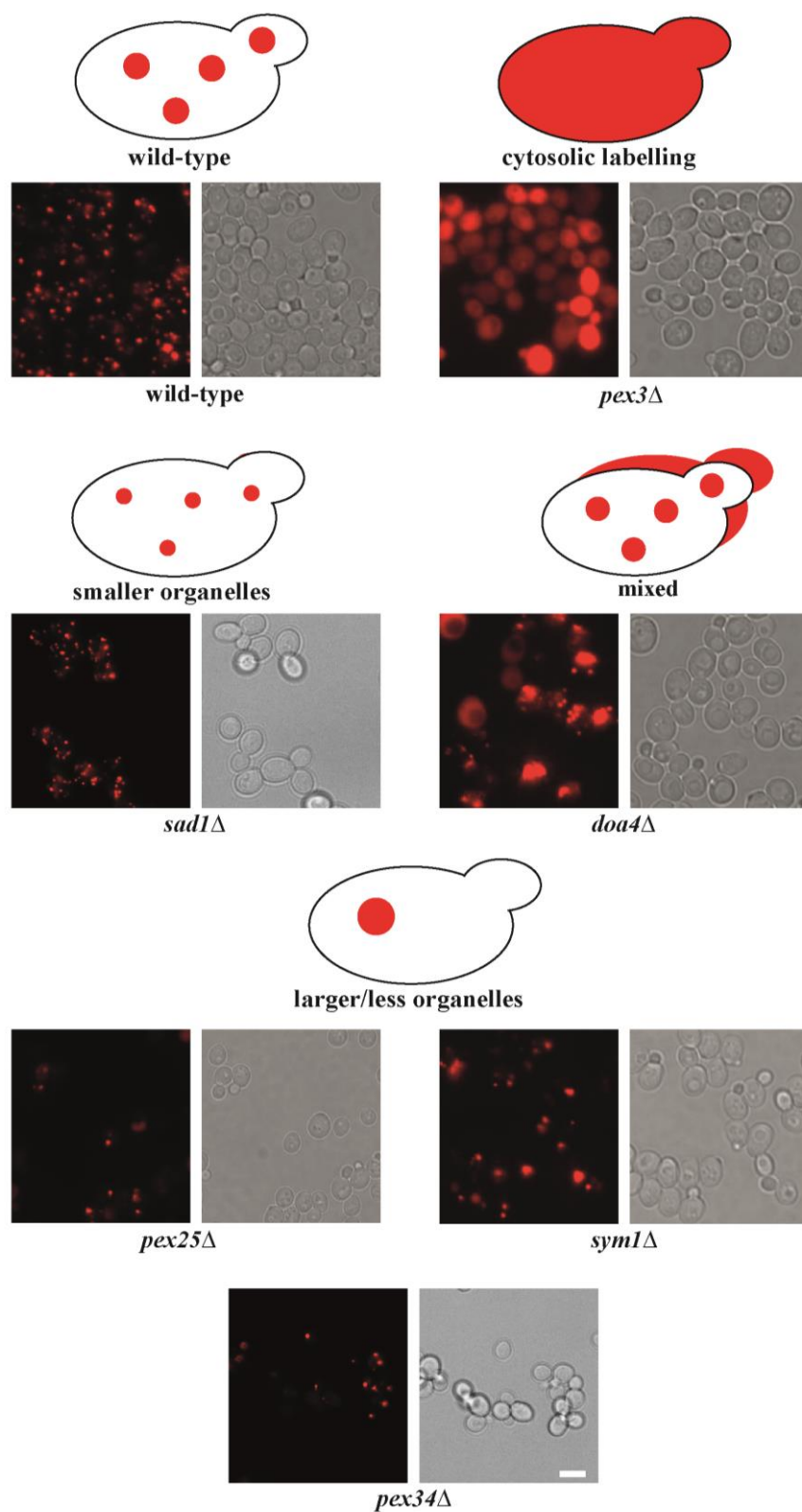


FIGURE 3

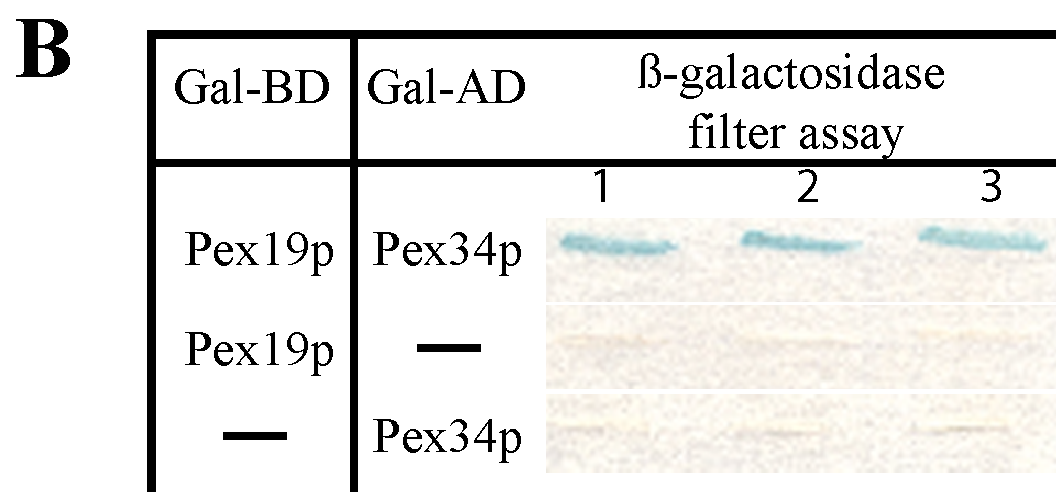
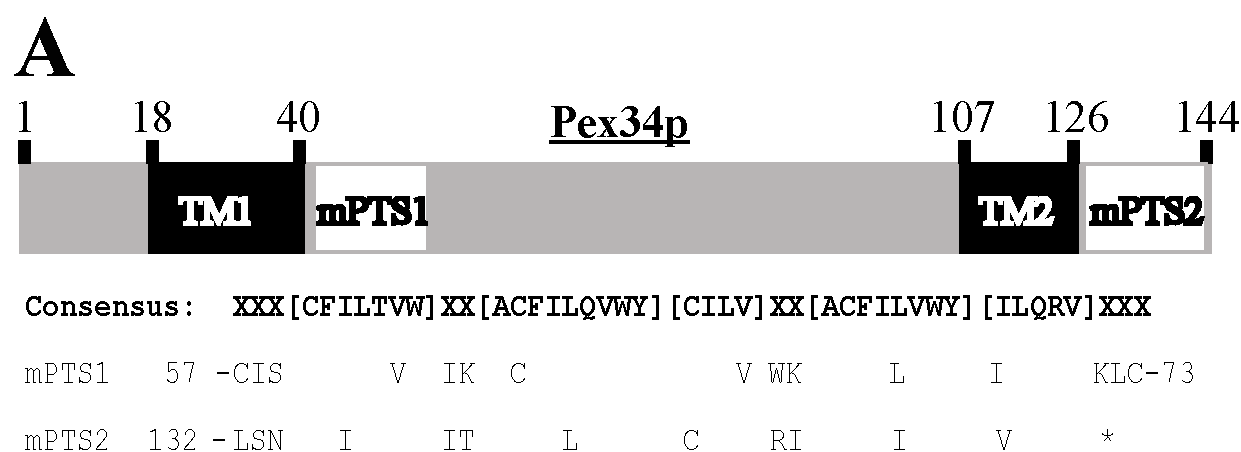


FIGURE 4

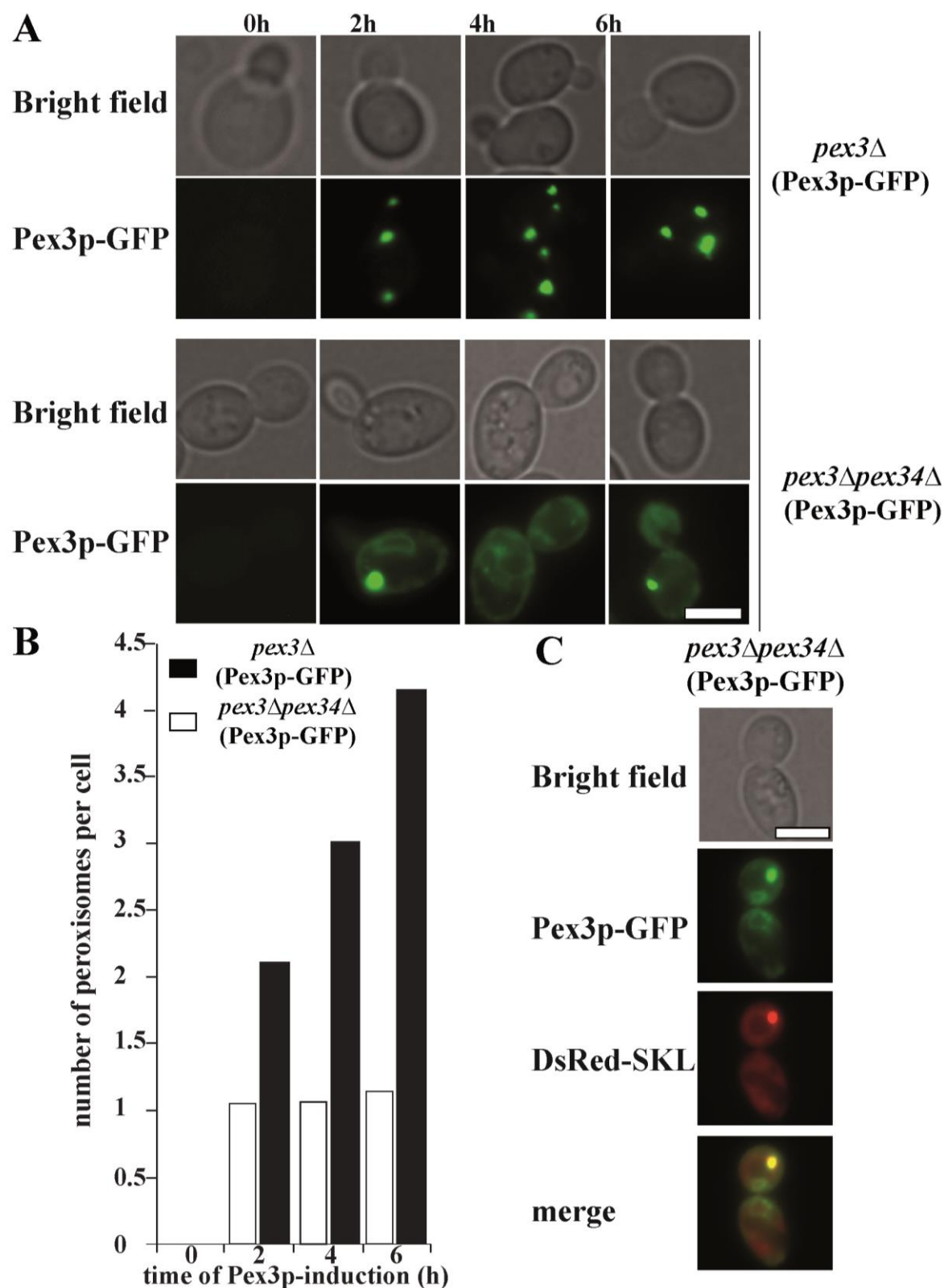


FIGURE 5

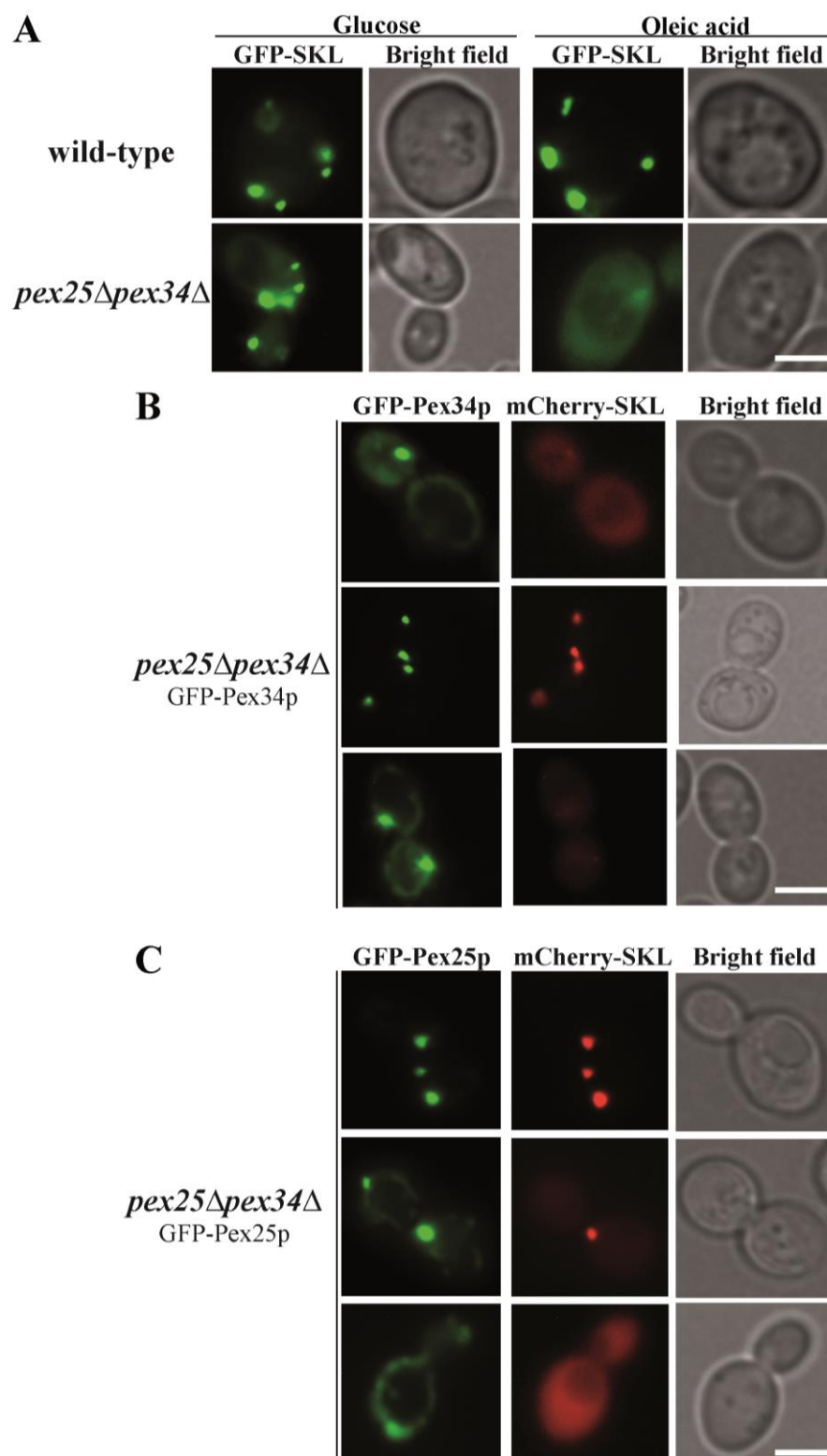


FIGURE 6