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The Phosphoinositide-3-Kinase Vps34p is required for pexophagy in *Saccharomyces cerevisiae*

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Abbreviations: PAS, pre-autophagosomal structures; PId, Phosphoinositides; PtdIns3P, Phosphatidylinositol 3-phosphate;

SYNOPSIS

Phosphoinositides are phosphorylated derivatives of the membrane phospholipid phosphatidylinositol (PtdIns) which have emerged as key regulators of many aspects of cellular physiology. We discovered a PtdIns3P synthesizing activity at peroxisomes from baker's yeast and demonstrate that the lipid kinase Vps34p is associated with peroxisomes already during biogenesis. However, Vps34 is required but is not essential for optimal peroxisome biogenesis. The function of Vps34p containing complex I as well as a subset of PtdIns3P binding proteins proved to be mandatory for the regulated degradation of peroxisomes, demonstrating that PtdIns3P-mediated signalling is required for pexophagy.

INTRODUCTION

Peroxisomes are single membrane bound subcellular organelles ubiquitously present in eukaryotic cells. Common metabolic functions of peroxisomes are the oxidation of fatty acids as well as the generation and removal of hydrogen peroxide. Peroxisomes have also been implicated in a row of other metabolic activities like synthesis of plasmalogens, isoprenoids, penicillin and lysine, and catabolism of polyamines, D-amino acids and methanol [1]. A remarkable property of peroxisomes is their unique variability in enzyme content and metabolic function that depends on cell or tissue type, organism and cellular needs [2]. The mechanism regulating the abundance of peroxisomes can be subdivided into different stages. When fermentable sugars are present, yeast β -oxidation genes are turned off by a mechanism termed glucose repression [3]. On non-fermentable carbon sources these genes become de-repressed resulting in an increase in the expression of genes including those coding for peroxisomal proteins [4]. Degradation of fatty acids in fungi and plants is exclusively localized in peroxisomes [5]. In baker's yeast growth on oleic acid as sole carbon source results in a striking proliferation of peroxisomes and is accompanied by a massive induction of genes coding for enzymes of the peroxisomal β -oxidation system [4]. When the peroxisome proliferation stimulus is removed and/or peroxisomal metabolism is no longer required, peroxisomal abundance is rapidly decreased by a selective autophagic process, termed pexophagy [6]. Interestingly, different lipids are involved in the regulation of this process. The peroxisomal induction is under the control of nuclear transcription factors regulated by lipid-derived peroxisome proliferators [4]. In *Sacharomyces cerevisiae*, oleic acid serves as a specific signal for peroxisomal proliferation and the transcriptional regulation of genes encoding peroxisomal functions is based on the oleate response element (ORE) [4]. Specific degradation of peroxisomes via pexophagy has been linked to PtdIns3P and PtdIns4P pathways as factors of the degradation machinery facilitating engulfment and subsequent degradation of peroxisomes in the vacuolar lumen contain phosphoinositide (PI)-binding domains [7]. However, PtdIns3P and PtdIns4P as well as the two identified pexophagy-specific binding proteins Atg24p and Atg26p have been shown to localize to autophagic membranes during pexophagy and not to peroxisomes themselves [8].

Here we report on our discovery that yeast peroxisomes are capable to synthesize PtdIns3P and demonstrate that Vps34p, the only PtdIns 3-kinase in baker's yeast is

localized at peroxisomes under inducing conditions. Cells lacking Vps34p are impaired in selective peroxisomal degradation, indicating that PtdIns3P signalling is required for the regulation of pexophagy.

EXPERIMENTAL

Strains and culture conditions – *S. cerevisiae* strains used in this study are listed in Table I. Genomic tagging of *VPS34* was performed according to [9] using primers RE1946 and RE1947 for the PCR. The *vps34Δ* strain was constructed by deletion of *VPS34* of the wild-type UTL7-A according to [9]. Transformants were selected for the appropriate marker and proper integration was confirmed by PCR. Yeast complete (YPD) and minimal media (SD) have been described previously [10]. Media contained 0.1% oleic acid and 0.05% Tween 40 (induction, YNO) or 2% glucose (repression) or 2% ethanol (de-repression) plus 0.1% yeast extract and 0.67% yeast nitrogen base without amino acids, adjusted to pH 6.0. For pexophagy studies, strains were grown on YNO medium over night. Cells were harvested, washed with sterile water and shifted to the pexophagy medium containing 2% glucose, 0.17% yeast nitrogen base without amino acids, adjusted to pH 6.0. When necessary, auxotrophic requirements were added according to [11].

Plasmids - The plasmid pAR2 coding for GFP-PTS1 [12] was used as a peroxisomal marker. Localization of GFP fusion proteins was also analyzed in strain yHPR251, which expresses PTS2-DsRed as peroxisomal marker from an integrated plasmid in strain UTL7A. For EGFP-tagging of Vps34p, the *VPS34* open reading frame was amplified by PCR with genomic *S. cerevisiae* DNA as template and RE1988 and RE1989 as primers. The *VPS34* PCR product was digested with *Sma*I/*Sal*I and cloned into pUG36. pGEX-6P-3 plasmids encoding the double FYVE-finger of mouse Hrs (aa 147-223) or its mutated form carrying a C215S mutation in both FYVE domains [13] were cut with *Eco*RI and *Sal*I. The isolated fragments were ligated into the *Eco*RI/*Sal*I digested pUG36, resulting in EGFP-2xFYVE and EGFP-2xFYVE(C215S).

Antibodies and Immunoblotting - Immunoblots were incubated with polyclonal rabbit antibodies raised against Pcs60p [14], Pex11p [15], ProtA (Sigma, St. Louis, MO), Kar2p [16], Pep12p (Molecular Probes, Leiden, Netherlands), ALP (Molecular Probes, Leiden, Netherlands), Gas1p [17], Fbp1p [18], Fox1p [19], Fox3p [20] and Pex13p [15]. Anti-rabbit coupled HRP (Sigma-Aldrich, Taufkirchen, Germany) was used as second antibody and blots were developed using the ECL-system (Amersham Buchler GmbH, Braunschweig, Germany).

Isolation of peroxisomes - Preparation of yeast spheroplasts, cell homogenization and determination of the suborganellar localization of proteins were performed according to [10]. For density gradient centrifugation, postnuclear supernatants (10 mg protein) were prepared and loaded onto preformed 2.25-24% (w/v) Optiprep (Iodixanol) gradients. Peroxisomes were separated from other organelles in a vertical rotor (Sorvall TV 860, 1.5 h at 48.000xg, 4°C). Fractions were collected from the bottom and subjected to enzyme and refractive index measurements as well as immunoblot analysis.

Radioactive labeling of cell organelles and chromatographic separations - The organelles from the gradient fractions were diluted in sucrose buffer and sedimented for 1 h at 100 000xg. Labeling, thin layer chromatography and the preparation of [³²P]-labeled PId standards were performed according to [21] and [22]. HPLC analysis of phosphoinositols was carried out according to [22] on a 250 - 4.6 mm Partisil SAX column (Whatman) by the use of a Merck-Hitachi LaChrom Elite HPLC system equipped with an Ramona Star solid scintillator radioflow detector (Raytest, Straubenhardt, Germany). The following gradient for elution of the HPLC column was used: buffer A contained distilled water, buffer B contained 1 M (NH₄)₂HPO₄ (pH 3.8). The gradient was run at 0% buffer B for 10 min and increased to 25% buffer B over 60 min and then to 100% buffer B over 20 min. The flow rate was 1 ml/min.

Miscellaneous - Whole cell yeast extracts were prepared from oleic acid-induced cells as described earlier [15]. GFP- and dsRed-tagged proteins were monitored by life cell imaging with a Zeiss Axioplan fluorescence microscope and AxioVision 4.1 software (Zeiss, Jena, Germany). The activity of catalase (EC 1.11.1.6) was determined according to [23].

RESULTS

S. cerevisiae peroxisomes contain Phosphoinositides - Peroxisomes isolated from rat liver harbor the PtdIns kinase activities for the formation of different PIDs such as PtdIns4P, PtdIns(4,5)P₂ and PtdIns(3,5)P₂ [21]. These findings prompted us to analyze whether also peroxisomes from baker's yeast possess the enzymatic capability to synthesize Ptds. As other endomembranes like endosomes, vacuoles, multivesicular bodies and the Golgi apparatus maintain this enzyme activity [24], separation of peroxisomes from these organelles was prerequisite for the analysis. To this end, organelles were separated from a cell free homogenate of oleic acid-induced wild-type cells by density gradient centrifugation, using a mixture of sucrose and Optiprep as gradient media. After centrifugation, 23 fractions were collected and subjected to the detection of organellar markers by enzyme measurement and immunoblot analysis. According to the localization of the peroxisomal markers catalase, thiolase (Fox3p), Pex11p and Pex13p, peroxisomes peaked at the bottom of the gradient at a high density of 1.21 g/cm³ well separated from mitochondria, vacuoles and endosomes (**Fig. 1A**). The presence of a smaller amount of thiolase in fractions with lower density is likely due to its release from broken organelles. The minor amount of the peroxisomal membrane markers Pex11p and Pex13p which is detected in lighter fractions is supposed to reflect the position of immature peroxisomes. The mitochondrial porin migrated to fractions of medium density. Vacuolar alkaline phosphatase (ALP, [25]) and endosomal Pep12p [26] were both found in the upper fractions of the gradient. The ER was distributed over a range of fractions as indicated by the detection of Kar2p [16] but well separated from the peroxisomal fractions.

To test their capability to synthesize PIDs, the organelles of the obtained gradient fractions were collected by centrifugation and subjected to [³²P]-ATP-labeling. Labeled phospholipids were extracted from the samples and analyzed by thin layer chromatography (**Fig. 1B**). Most of the synthesized PIDs appeared in the gradient fractions 19 to 21 predominantly containing endosomal membranes (**Fig.1B**). Significant

amounts of newly synthesized PIDs were also observed in fractions containing mitochondria and ER. Most interestingly, in the high density peroxisomal fractions PID-synthesizing activity was also detected (*Fig. 1B, lane 3*). To identify the nature of these peroxisomal PIDs, the labeled phospholipids were extracted, deacylated and separated by HPLC (**Fig. 1C, upper panel**). For clear identification, the samples were supplemented with PtdIns3P or PtdIns4P standards that were added prior to the HPLC (**Fig. 1C, middle and lower panel**). Comparison of the elution profiles of peroxisomal PIDs with those of the external PID standards revealed that PtdIns3P is the major product generated by peroxisome labelling.

A portion of the lipid kinase Vps34p localizes to peroxisomes – Our results demonstrate that yeast peroxisomes possess the ability to synthesize PtdIns3P and thus are associated with a PtdIns 3-kinase. The only PtdIns 3-kinase known in baker's yeast is Vps34p, which has been shown to synthesize PtdIns3P at vacuolar and endosomal membranes [27, 28]. To investigate whether Vps34p is also responsible for the peroxisomal synthesis of PtdIns3P, the subcellular localization of the protein was analyzed under peroxisome-inducing conditions. First, we tested for co-localization of GFP-tagged Vps34p with peroxisomes by fluorescence microscopy (**Fig. 2A**). GFP alone or GFP fused to the N-terminal part of Vps34p were co-expressed in wild-type cells together with the PTS2-DsRed, which served as a peroxisomal marker. GFP alone led to an overall cytosolic staining while GFP-Vps34p exhibited a weak cytosolic but clear punctuate pattern that co-localized with the PTS2-DsRed staining, demonstrating that the fusion protein localizes at least in part to peroxisomes (**Fig. 2A**). These observations were corroborated by subcellular fractionation studies. A cell free homogenate of oleic acid-induced wild-type cells expressing a genomically tagged *VPS34* gene coding for a Vps34p-protein A fusion protein (Vps34p-TEV-ProtA) was prepared and organelles were separated by density gradient centrifugation (**Fig. 2B**). As judged by immunoblot analysis of the obtained fractions, peroxisomes predominantly migrated to the bottom fractions indicated by the presence of the peroxisomal marker protein Pcs60p. In contrast, the mitochondrial marker (porin) and the ER marker (Kar2p) were both found in the middle of the gradient, clearly separated from the peroxisomal peak fraction. Vacuolar ALP and endosomal Pep12p as well as Gas1p, a marker protein of the plasma membrane [17], remained in the top fractions of the gradient. In line with the well known cytosolic, vacuolar and endosomal localization, the majority of Vps34p remained in the top gradient fractions. However, a significant portion of Vps34p was detected at a higher density, co-localizing with peroxisomal Pcs60p (*Fig. 2B, lane 3*). Taken together, the localization studies demonstrate that a significant portion of Vps34 is associated with peroxisomes.

Vps34p is not required for peroxisome biogenesis – Vps34p is in control of several pathways in membrane trafficking and organelle formation. Thus, a requirement of Vps34p for peroxisome formation was considered and investigated. To this end, cell homogenates were prepared from the *vps34Δ*-mutant strain and organelles were separated by density gradient centrifugation as outlined in **Fig. 3A**. The majority of the peroxisomal marker Pcs60p was detected in fractions of high density as typical for mature peroxisomes, indicating that formation of peroxisomes seems not to be disturbed. As a next step, the *vps34Δ*-strain was analyzed for the peroxisomal targeting of GFP fused to

the peroxisomal targeting signal 1 (GFP-PTS1). Fluorescence microscopy analysis of oleic acid-induced live cells revealed a punctuate staining pattern in the wild-type strain that is typical for peroxisomal labeling (**Fig. 3B**). Expression of GFP-SKL in *pex5Δ* cells that are affected in peroxisomal protein import exhibited a cytosolic fluorescence pattern as typical for these cells. The fluorescence microscopy pattern observed for the *vps34Δ* strain was similar to the one visible in the wild-type strain, suggesting that *vps34Δ* cells are still able to import peroxisomal matrix proteins.

Peroxisomes are required for growth of baker's yeast on fatty acids as single carbon source but are dispensable for growth on other fermentable and non-fermentable carbon sources. In order to test whether Vps34p is required for peroxisome function, the growth abilities of *vps34Δ* cells on either glucose or oleic acid as carbon source were analyzed. As the *vps34* mutant was described to be temperature sensitive [29], growth tests were performed at different temperatures. As shown in **Fig. 3C**, all strains were capable in utilizing glucose as sole carbon source at 26°C and 30°C. In line with temperature sensitivity, *vps34Δ* exhibited significantly reduced growth behaviour at the restrictive temperature of 37°C (data not shown). When oleic acid served as sole carbon source, the wild-type cells grew at all temperatures. In contrast, the *pex3Δ* strain, defective in peroxisome biogenesis, was unable to grow on this medium, as typical for these cells [10]. At 26°C, *vps34Δ* still grew on oleic acid medium although slower than the wild-type, however, at 30°C the cells exhibited a severe growth defect on oleic acid medium suggesting that they are affected in peroxisome function. As peroxisomes were present, indicating that the biogenesis of the organelles seemed not to be affected, the peroxisomal enzyme composition was analyzed in more detail. We monitored the level of the peroxisomal marker enzyme Fox3p by immunoblot analysis of lysates derived from wild-type, *vps34Δ* and *vps15Δ* cultures. Fox3p was not detectable in cells grown in 2 % glucose medium, which is in agreement with the fact that the *FOX3* locus is glucose-repressed (**Fig. 3D**). After shift to oleate-medium, the samples derived from the *vps34Δ* and *vps15Δ* strains contained only a small amount of Fox3p compared to wild-type (data not shown). However, we also noticed that the two deletion strains grew significantly slower than the wild-type. After adjustment of the generation time (wild-type: 14 h; deletion strains: 28 h), we found that the Fox3p level in *vps34Δ* and *vps15Δ* cells was comparable to the wild-type strain (**Fig. 3D**). The mitochondrial porin served as loading control. Taken together, these results indicate that Vps34p function is not essential for peroxisome biogenesis but has an impact on the growth rate of the cells.

Vps34p complex I is involved in degradation of peroxisomes – S. cerevisiae adjusts the level of peroxisomes and peroxisomal enzymes in response to differing nutritional sources. When cells are grown on oleic acid as the sole carbon source, proliferation of peroxisomes and synthesis of peroxisomal enzymes is induced. Conversely, a subsequent shift to glucose-rich or nitrogen-limiting conditions results in regulated peroxisome degradation (pexophagy) requiring the activity of vacuolar hydrolases [6]. Deletion of the putative Vps34p orthologue Pdd1p from *H. polymorpha* affects peroxisome degradation and the sorting of the vacuolar proteinase HpCPY [30]. Based on the assumption that Vps34p may carry out a similar function, we analyzed the selective degradation of peroxisomes in presence and absence of Vps34p. Cells were grown on oleic acid medium and subsequently shifted to glucose rich medium to induce pexophagy. Samples were

taken at different time points and whole cell lysates were prepared and analyzed by immunoblot analysis. Degradation of peroxisomal thiolase within 24h served as a marker for functional pexophagy. Under these conditions, the steady-state concentration of mitochondrial porin was not affected demonstrating that the experimental setup is suitable to specifically study the degradation of peroxisomes (**Fig. 4A**). Pep4p is a vacuolar aspartyl protease and required for the posttranslational precursor maturation of vacuolar proteinases [31]. Deletion of this protease severely affects vacuolar degradation of Fox3p that is still visible after 24 h. Interestingly, in cells lacking Vps34p, a significant amount of thiolase was still present after 24 h on glucose medium. These results demonstrate that peroxisomal degradation was slowed down and that Vps34p plays a role in pexophagy in baker's yeast. In contrast, the deletion of the gene encoding for the PtdIns3P 5-kinase Fab1p had no effect on pexophagy. This finding indicates that PtdIns3P is not required as an intermediate for the generation of PtdIns(3,5)P₂ but itself represents the crucial signalling lipid required for pexophagy.

In yeast, Vps34p is part of two separate protein complexes [8]. Both complexes contain Vps15p and Vps30p(Atg6) associated with Vps34p. Complex I is functionally connected to autophagy via its specific component Atg14p. Complex II contains Vps38p instead of Atg14p and is required for the sorting of vacuolar proteins. While the single deletions of Vps15p, Vps30p and Atg14p resulted in a block of pexophagic degradation, the deletion of Vps38p did not influence this process (**Fig. 4B**). The finding that Atg14p but not Vps38p is required for the autophagic removal of peroxisomes indicates that the PtdIns3P generated by complex I is required for this process.

Next we analyzed by which principle mechanism Vps34p may regulate pexophagy via its product PtdIns3P. In principle, accumulation of PtdIns3P itself could be required to define a certain membrane topology (discussed in [32]). The second possibility is that it recruits a subset of PtdIns3P-binding proteins. Several of such PtdIns3P effector proteins have been described in different species. Most of these effector proteins interact with PtdIns3P via the FYVE (conserved in Fab1, YOTB, Vac1 and EEA1) zinc finger domain [33] or via the PX (Phox-homology) domain [34, 35]. We transformed cells expressing PTS2-DsRed with either EGFP-2xFYVE or EGFP-2xFYVE(C215S) and monitored their localization in oleate-grown cells by fluorescence microscopy (**Fig. 4C**). The EGFP-2xFYVE expression generated a punctuate pattern. These signals co-localized in most cases with the peroxisomal marker PTS2-DsRed. In marked contrast, the EGFP-2xFYVE(C215S) mutant, which is affected in its capacity to bind PtdIns3P [13, 33], displays no co-localization with the peroxisomal marker, demonstrating the specificity of the previously observed association. Using an alternative method, this result provides further evidence for the specific localization of PtdIns3P to peroxisomes in oleate-induced cells and thus adds up to the data presented in Fig. 1. In addition, the specific localization of the FYVE-probe to PtdIns3P-containing peroxisomal structures indicates that the peroxisomal PtdIns3P pool is accessible to PtdIns3P binding module containing proteins. It has been described that overexpression of the FYVE-domain is capable to disrupt PtdIns3P-mediated functions, most likely by competing with PtdIns3P-binding proteins [36]. In order to investigate, if PtdIns3P itself or if PtdIns3P-effector mediated signalling is required for pexophagy, we overexpressed the double FYVE domain or alternatively the corresponding C215S mutant (**Fig. 4D**). Similar to the assay described above, the oleate-grown cells were shifted to glucose-medium to induce pexophagy. The

overexpression of the FYVE-domain inhibited the degradation of Fox3p, while the FYVE(C215S) mutant was not affected in the decrease of the Fox3p level. These data indicate Vps34p regulates pexophagy via PtdIns3P effectors that are most likely directed to the peroxisomal pool of PtdIns3P.

Identification of down-stream effectors of PtdIns3P. The proteome of *S. cerevisiae* contains five proteins harbouring a FYVE-domain which exclusively binds PtdIns3P. Furthermore, yeast has 15 proteins containing a PX-domain which enables the recognition of PtdIns3P as well as other types of PtdInsP. In addition, eight other proteins have been published to interact with PtdIns3P via an non-canonical interaction motive [37]. In order to identify down-stream effectors of PtdIns3P, we screened the corresponding 28 deletion strains for a defect in pexophagy (Fig. 5). We found eleven deletion strains to display a delayed degradation of Fox3p or an almost block of Fox3p breakdown.

Previous data already established a role for the *S. cerevisiae* Atg proteins Atg18p, Atg20p, Atg24p and Atg27p in pexophagy [8], while we show here for the first time that the homolog of the *H. polymorpha* Vam7p [38], a vacuolar SNARE-complex protein, is essential for this process in *S. cerevisiae*. Equally novel new is the principal connection of six other proteins to the autophagic degradation of peroxisomes. The deletion strains *bem3Δ*, *mvp1Δ* and *vps27Δ* show a slightly delayed degradation of Fox3p, while the deletion strains *arc1Δ*, *bem1Δ* and *pep7Δ* show a significantly reduced breakdown of Fox3p (Fig. 5). Pep7p, Vps27p and Mvp1 are connected to membrane traffic and protein sorting between different cellular compartments, while Arc1p is a tRNA binding protein and Bem1p as well as Bem3p are connected to Cdc42p-mediated signalling. The functional implication of these proteins may give first indication of a connection, either directly or indirectly, of the mentioned processes to the autophagic degradation of peroxisomes.

DISCUSSION

Here we report on the detection of a PtdIns3P-synthesizing activity associated with peroxisomes from baker's yeast and the identification of Vps34p as the responsible kinase. We show that Vps34p is associated with peroxisomes under inducing conditions and provide evidence that the protein is vital for the controlled degradation of peroxisomes by pexophagy.

Previous findings have demonstrated that peroxisomal membranes contain phosphoinositides (PIs) [21, 39]. In rat liver peroxisomes, PtdIns is used as a substrate and converted to PtdIns4P, PtdIns(3,5)P₂ and PtdIns(4,5)P₂ [21]. However, although some PtdIns3P was detected *in vivo* on peroxisomes, it was hardly detected *in vitro*, raising the question if peroxisomes themselves are competent to harbour the enzyme activity required for its synthesis or not. Here, we identified the synthesis of PtdIns3P at the peroxisomal membrane of baker's yeast (Fig. 1). In yeast, Vps34p is the only known kinase which phosphorylates PtdIns at the 3' hydroxyl position [40]. Vps34p and its orthologues from different species have been implicated in the control of important pathways, including the endosome to lysosome transport, multivesicular body formation,

and autophagy. Accordingly, the enzymes and their products have been localized to endosomal and vacuolar membranes and were detected on pre-autophagosomal structures (PAS) [8]. While it has been known that Vps34p is associated with autophagic and endosomal membrane systems [41], we now demonstrate that a significant portion of Vps34p is associated also with peroxisomes (Fig. 2). The reason why peroxisomal localization has not been observed previously most likely is due to the fact that former localization studies were performed with glucose-grown cells possessing peroxisomes in their non-induced state. This property is not unique for Vps34p, as for example, the small G-protein Rho1p is predominantly tethered to membranes of the secretory pathway, but is specifically recruited to peroxisomes upon their induction [42]. Interestingly, besides to endosomes, vacuoles and PAS, Vps34p was also localized to dot like structures considered representing a novel undefined compartment [43] that in the light of our data might represent peroxisomes.

Interestingly, we found Vps34p to localize to peroxisomes already during biogenesis of peroxisomes, raising the question if Vps34p is involved in this process. However, the GFP-PTS1 cargo could still be imported efficiently into the peroxisomal matrix in the *vps34Δ* background. Although the growth of the *vps34Δ* strain was significantly slower on oleate plates compared to the wild-type strain, the deletion of *VPS34* proved not to be essential for peroxisome biogenesis. One explanation for the slower growth on oleate may come from a recent report [44]. It was suggested that Vps34p is involved in the derepression of oleate-inducible genes. This result was obtained in a screen for mutant strains that were affected in glucose repression, glycerol derepression or oleate induction of the *POT1* locus. Using FACS to monitor the expression level of Pot1p-GFP, [44] found that the deletion of *VPS34* had a significant effect on the transition from the glucose-repressed state to either glycerol-derepressed or oleate-induced states as judged by reduced signal intensity of the Pot1p-GFP probe. We obtained similar results when we monitored the level of Fox3p by immunoblotting. While Fox3p was strongly induced in wild-type cells after shift from glucose to oleate-medium, the *vps34Δ* strain contained only little Fox3p. However, we also noted that the cells themselves grew with an about 50 % slower rate than the wild-type cells. As the amount of Fox3p was similar in all strains after adjustment of the generation time, we conclude that Vps34p may not be directly involved in the derepression of oleate inducible genes but seems to have a more pleiotropic effect on cell growth and the rate of organellar biogenesis. In addition, the fact that the *vps34Δ* strain also grows slower on glucose medium points to a more pleiotropic effect.

Originally, Vps34p was discovered as a gene required for sorting of carboxypeptidase Y (CPY) to the vacuole [29]. In line with this function, a number of phenotypes were described that were related to vacuolar malfunctions. These include temperature-sensitive growth, disturbed vacuolar segregation and missorting of a number of soluble vacuolar proteinases resulting in impaired vacuolar degradation. Experiments designed to isolate mutants specifically affected in peroxisome degradation in *H. polymorpha* identified Pdd1p, a putative orthologue of Vps34p [30]. The *pddl* mutant is blocked in the initial sequestration of peroxisome degradation during either glucose or ethanol adaptation. We show that in baker's yeast Vps34p is required for the regulated degradation of peroxisomes by pexophagy as demonstrated by the reduced disappearance of the peroxisomal marker protein Fox3p (thiolase) in *vps34Δ* cells after shifting from oleate

induction to glucose medium. (Fig. 4A). In addition, we found that Vps34p complex I is essential for pexophagy, which contains the autophagy-specific component Atg14p, while Vps38p-containing complex II is dispensable. This finding is novel, although it should be noted that a recent publication in *P. pastoris* shows that the $\Delta vps38$ strain is not affected in pexophagy [45].

In this study, we performed a screen for down-stream effectors of Vps34p-function (Fig. 5). The PtdIns3P-binding Atg proteins Atg18p, Atg20p, Atg24p and Atg27p of *S. cerevisiae* have been linked to pexophagy previously [8]. Considering the species-specific differences of the pexophagy machinery, we confirmed that Atg21p, which is essential for pexophagy in *H. polymorpha* ([46], is dispensable for this process in *S. cerevisiae*, as noticed before [47]. In this context, it is interesting to note that the *H. polymorpha* homolog of Vam7p, which is part of the vacuolar SNARE complex [38], turned out to be essential for pexophagy in *S. cerevisiae*. In addition, we could show that some deletion strains are affected in pexophagy, which have not been linked before to this process or even autophagy-related pathways in general.

The deletion strains lacking Vps27p or Mvp1p exhibit a mild defect in pexophagy by displaying a slightly delayed degradation of Fox3p. This might indicate that their association with pexophagy is the modulation of this process or the association is indirect by affecting more general sorting pathways. Vps27p is part of the endosomal ESCRT-machinery which is required for the biogenesis of multivesicular bodies and the sorting of ubiquitinated proteins to the vacuole, but it is also involved in the maintenance of the vacuolar morphology. It has been shown that the delivery of certain hydrolases to the vacuole is perturbed in yeast ESCRT mutants [48]. Mvp1p is a SH3-domain containing protein that acts in concert with the dynamin-like GTPase Vps1p to promote membrane traffic to the vacuole [49]. Vps1p has also been shown to regulate peroxisome abundance, inheritance and also fission [50, 51]. The dynamin-like GTPase Dnm1p is part of the peroxisomal as well as mitochondrial fission machinery [51, 52] and it is interesting to note that $\Delta dnm1$ cells are affected in mitophagy [53].

The mutant strains lacking Pex7p, Arc1p, Bem1p or Bem3p exhibit a severe defect in pexophagy, indicating that these proteins might play an important role for this process.

Pep7p is a multivalent adaptor protein involved in vesicle-mediated vacuolar protein sorting and vacuolar inheritance [54]. Another possible connection to autophagic processes is its interaction to the t-SNARE Pep12p [55], which is required for selective autophagy of mitochondria [53]

Arc1p has been found to bind to tRNA and is supposed to function as a cofactor of methyl- and glutamyl-tRNA synthases [56]. It is interesting to note Arc1p is supposed to be target of the ser/thr kinase Atg1p [57], which is required for early steps of autophagic processes, including pexophagy [58].

Work on Bem1p has focused on its role as scaffold protein for complexes of the essential GTPase Cdc42p with its guanine nucleotide exchange factor (GEF) Cdc24p and their functional role in cell polarity, assembly of the bud emergence site as well as reorganization of the actin cytoskeleton [59]. Further data indicate a role of Bem1p as positive regulator of homotypic fusion of yeast vacuoles. [60]. Other possible links to autophagic degradation of peroxisomes might be indicated by association of Bem1p with actin, Vac8p as well as Vam7p [60] because these proteins are also required for pexophagy [6]. Bem3p is another interaction partner of Bem1p, and *bem3* Δ cells also

proved to be affected in pexophagy. Bem3p is a GTPase activating protein (GAP) for Cdc42p, hinting to a second possible link between Cdc42p-complexes and autophagic degradation of peroxisomes.

Clearly, further work is required to fully analyze the functional connection of the tested proteins to selective autophagy of peroxisomes. It will be important to elucidate, which autophagy-related pathways and membrane-trafficking pathways are interconnected by these proteins and thus at which step, be it specific or indirectly, they influence pexophagy.

The mechanisms by which Vps34p and its product PtdIns3P regulate pexophagy are still incompletely understood. In line with the finding that Vps34p function is required at an early stage in autophagy, yeast cells affected in *VPS34* seem to block pexophagy at very early stage as judged by electron microscopy [30]. Previous reports demonstrate that PtdIns3P is present at the PAS as well as on the vertex and boundary of the pexophagosome/vacuole fusion site [61] during pexophagy in *P. pastoris*. The finding that Vps34p and PtdIns3P do not only localize to autophagic membranes but also to the potential target of selective autophagy raises the possibility that the formation of the initiation may start at the target itself.

Our data are clear in that yeast peroxisomes functionally recruit Vps34p leading to the formation of PtdIns3P, which is required for pexophagy. However, the fact that rat liver peroxisomes besides PtdIns3P synthesize other distinct PIDs raises the question whether PtdIns3P is the only PID synthesized in the yeast peroxisomal membrane. The discovery that Fab1p, a PtdIns3P 5-kinase, is required for growth on oleic acid [62] opens the possibility that the peroxisomal PtdIns3P might be processed further. However, our data show that Fab1p is not required for pexophagy (Fig. 4A), indicating that PIDs might play a more complex role in the regulation of peroxisome function and biogenesis.

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Table I Strains used in this study

Strain	Description	Source or Reference
UTL7-A	<i>MATα leu2-3, 112 ura3-52 trp1</i>	[10]
<i>pex3</i> Δ	UTL7-A; <i>pex3</i> :: <i>loxP</i>	this study
<i>vps34</i> Δ	UTL7-A; <i>vps34</i> :: <i>loxP</i>	this study
<i>fab1</i> Δ	UTL7-A; <i>fab1</i> :: <i>loxP</i>	this study
PTS2-DsRed (yHPR 251)	UTL7-A + <i>EcoRV</i> linearized pHPR131	[63]
BY4742	<i>MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0</i>	Euroscarf, Frankfurt, Germany
<i>arc1</i> Δ	BY4742; <i>arc1</i> :: <i>kanMX4</i>	Euroscarf
<i>atg14</i> Δ	BY4742; <i>atg14</i> :: <i>kanMX4</i>	Euroscarf
<i>atg18</i> Δ	BY4742; <i>atg18</i> :: <i>kanMX4</i>	Euroscarf
<i>atg20</i> Δ	BY4742; <i>atg20</i> :: <i>kanMX4</i>	Euroscarf
<i>atg21</i> Δ	BY4742; <i>atg21</i> :: <i>kanMX4</i>	Euroscarf
<i>atg24</i> Δ	BY4742; <i>atg24</i> :: <i>kanMX4</i>	Euroscarf
<i>atg27</i> Δ	BY4742; <i>atg27</i> :: <i>kanMX4</i>	Euroscarf
<i>bem1</i> Δ	BY4742; <i>bem1</i> :: <i>kanMX4</i>	Euroscarf
<i>bem3</i> Δ	BY4742; <i>bem3</i> :: <i>kanMX4</i>	Euroscarf
<i>egd2</i> Δ	BY4742; <i>egd2</i> :: <i>kanMX4</i>	Euroscarf
<i>fab1</i> Δ	BY4742; <i>fab1</i> :: <i>kanMX4</i>	Euroscarf
<i>hsv2</i> Δ	BY4742; <i>hsv2</i> :: <i>kanMX4</i>	Euroscarf
<i>mdm1</i> Δ	BY4742; <i>mdm1</i> :: <i>kanMX4</i>	Euroscarf
<i>mvp1</i> Δ	BY4742; <i>mvp1</i> :: <i>kanMX4</i>	Euroscarf
<i>pep7</i> Δ	BY4742; <i>pep7</i> :: <i>kanMX4</i>	Euroscarf
<i>pib1</i> Δ	BY4742; <i>pib1</i> :: <i>kanMX4</i>	Euroscarf
<i>pib2</i> Δ	BY4742; <i>pib2</i> :: <i>kanMX4</i>	Euroscarf
<i>rgd1</i> Δ	BY4742; <i>rgd1</i> :: <i>kanMX4</i>	Euroscarf
<i>snx3</i> Δ	BY4742; <i>snx3</i> :: <i>kanMX4</i>	Euroscarf
<i>snx41</i> Δ	BY4742; <i>snx41</i> :: <i>kanMX4</i>	Euroscarf
<i>spo14</i> Δ	BY4742; <i>spo14</i> :: <i>kanMX4</i>	Euroscarf
<i>vps5</i> Δ	BY4742; <i>vps5</i> :: <i>kanMX4</i>	Euroscarf
<i>vps15</i> Δ	BY4742; <i>vps15</i> :: <i>kanMX4</i>	Euroscarf
<i>vps17</i> Δ	BY4742; <i>vps17</i> :: <i>kanMX4</i>	Euroscarf
<i>vps27</i> Δ	BY4742; <i>vps27</i> :: <i>kanMX4</i>	Euroscarf
<i>vps30</i> Δ	BY4742; <i>vps30</i> :: <i>kanMX4</i>	Euroscarf
<i>vps34</i> Δ	BY4742; <i>vps34</i> :: <i>kanMX4</i>	Euroscarf
<i>vps38</i> Δ	BY4742; <i>vps38</i> :: <i>kanMX4</i>	Euroscarf
<i>ykr078w</i> Δ	BY4742; <i>ykr078w</i> :: <i>kanMX4</i>	Euroscarf
<i>ypr097w</i> Δ	BY4742; <i>ypr097w</i> :: <i>kanMX4</i>	Euroscarf

<i>ypt35Δ</i>	<i>BY4742; ypt35 ::kanMX4</i>	Euroscarf
<i>BY4743</i>	<i>MATa/MATα, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, met15Δ0/+, +/lys2Δ0, ura3Δ0/ura3Δ0</i>	Euroscarf
<i>cdc33Δ</i>	<i>BY4743; cdc33 ::kanMX4</i>	Euroscarf

Table II Oligonucleotides used in this study

Oligo-nucleotide	Sequence
RE 1988	5'GCCCCGGGATGTCACTGAACAACATAACATTC'3
RE 1989	5'GCGTCTGACTCAGGTCCGCCAGTATTGTTGC'3
RE 1936	5'AATAACATCTCCGTGAAGCATTGAGGGAAGGGTTTAACTCCCA GCTGAAGCTTCGTACGCT'3
RE 1937	5'GAGTGACGAAATTTAAATTTTGAAGCACCAATTATCAACCATAG GCCACTAGTGGATCTG'3
RE 1946	5'TGATTGATCATTACATAATCTGGCACAATACTGGCGGACCCGT ACGCTGCAGGTTCGAC'3
RE 1947	5'GAGTGACGAAATTTAAATTTTGAAGCACCAATTATCAACCAATC GATGAATTTCGAGCTCG'3
Ku 575	5'CGTAAAAGCAGAAGCACGAAACAAGGAGGCAAACCACTAAAA GGCGTACGCTGCAGGTTCGAC'3
Ku 862	5'CATGTTACCTATTGCACACTTACTGTATTAAAGATTACGCATAG GCCACTAGTGGATCTG'3

FIGURE LEGENDS

Fig. 1: Peroxisomes contain a PtdIns3P-synthesizing activity

Organelles from a cell free homogenate of oleic acid-induced wild-type yeast cells were separated by density gradient centrifugation (2.25%-24% Optiprep, 18% sucrose) and fractions were subjected to (A) measurement of the activity of catalase used as peroxisomal marker or immunoblot analysis with antibodies against the following marker proteins: Fox1p, Pex11p and Pex13p (peroxisomes); Porin (mitochondria); Kar2p (ER); ALP (vacuole); Pep12p (late endosomes); Fbp1p (cytosol). (B) Organelles from selected fractions were analyzed for Ptd-synthesizing activities by *in vitro* labelling with [γ - 32 P]ATP followed by thin layer chromatography of the extracted Ptds (C). Ptds synthesized *in vitro* by the peroxisomal fractions (3 and 4) were further analyzed by HPLC (upper panel). For the identification of the synthesized Ptds, PtdIns3P (middle panel) or PtdIns4P (lower panel) were added to the samples as external standards and analyzed as above. By comparison with standard Ptds, PtdIns3P was identified to be synthesized by peroxisomes.

Fig. 2: Vps34p is associated with peroxisomes

Localization studies were performed with a GFP- or TEV-ProtA-tagged Vps34p. (A) Plasmid-encoded GFP or GFP fused to Vps34p were co-expressed with the peroxisomal marker PTS2-DsRed in wild-type cells. Transformants were grown on oleic acid plates for 48 h and analyzed by fluorescence microscopy. Non-tagged GFP exhibits an overall cytosolic labelling, whereas GFP-Vps34p in addition leads to a punctuate staining pattern which to a significant extent co-localizes with the peroxisomal marker PTS2-DsRed. Bar, 10 μ m. (B) A cell free homogenate of oleic acid-induced wild-type cells expressing plasmid encoded Vps34p-ProtA was loaded onto a linear 2.25%-24% Optiprep gradient containing 18% sucrose. Fractions were subjected to immunoblot analysis with antibodies against the following marker proteins: Pcs60p (peroxisomes); Porin (mitochondria); Kar2p (ER); ALP (vacuole); Pep12p (late endosomes); Gas1p (plasma membrane). The result indicates Vps34p is predominantly detected in vacuolar and endosomal fractions; however, a significant portion is localized to peroxisomes.

Fig. 3: *vps34* Δ cells are not affected in peroxisome biogenesis but exhibit a defect in peroxisome function

(A) Peroxisomes of oleic acid-induced *vps34* Δ cells were isolated by density gradient fractionation as described for wild-type cells. Gradient fractions obtained were subjected to immunoblot analysis. Migration of the peroxisomal marker protein Pcs60p to high-density fractions demonstrates the presence of mature peroxisomes and indicates that deficiency in Vps34p does not severely affect peroxisome biogenesis. (B) The PTS1 marker protein GFP-PTS1 was transformed in wild-type, *pex5* Δ and *vps34* Δ -cells. The transformed strains were grown on oleic acid plates for two days and examined by fluorescence microscopy. In contrast to *pex5* Δ cells which are impaired in PTS1-dependent matrix protein import and mislocalized the marker protein to the cytosol, both wild-type and *vps34* Δ exhibit a punctuate GFP staining. These results demonstrate that the marker protein localizes to peroxisomes, excluding defects in peroxisomal protein import. Bar 2.5 μ m. (C) Indicated strains were spotted as a series of 10-fold dilutions on

media containing either glucose or oleic acid as sole carbon source and incubated for 5 days at 26 and 30°C as indicated. All strains were capable to utilize glucose as sole carbon source at 26°C and 30°C. On oleic acid medium, although slower than the wild-type, *vps34Δ* still grew at 26°C. At 30°C *vps34Δ* exhibited a severe growth defect on oleic acid medium, suggesting that the cells are affected in peroxisome function.

Fig. 4: Vps34p and PtdIns3P mediated signalling are required for pexophagy

(A) Strains were grown on oleic acid media for 16 h and subsequently shifted to glucose containing medium inducing pexophagy. After distinct periods samples were taken, whole cell lysates prepared and subjected to SDS-PAGE and immunoblot analysis. Mitochondrial porin served as an internal control. Wild-type cells degraded Fox3p (peroxisomal ketoacyl-CoA thiolase) and thus peroxisomes were considered to be degraded via pexophagy within 24 hours while *vps34Δ* a significantly reduced degradation rate, indicative for defective pexophagy. *pep4Δ* known to be affected in pexophagy served as control. (B) In order to analyze whether Vps34 complex I (Vps15p/Vps30p/Atg14p) or complex II (Vps15p/Vps30p/Vps38p) is involved in pexophagy the corresponding deletion strains were tested as described above. (C) Plasmid-encoded EGFP-2xFYVE or EGFP-2xFYVE(C215S) were co-expressed with the peroxisomal marker PTS2-DsRed in wild-type cells. Transformants were grown on oleic acid plates for 48 h and analyzed by fluorescence microscopy. The EGFP-2xFYVE displays an overall a punctuate pattern which co-localizes to a significant extent with the peroxisomal marker PTS2-DsRed, whereas the mutant form of the FYVE-domain does not co-localize with the peroxisomal marker. (Bar, 10 μm.). (D) Pexophagy was monitored in cells constitutively expressing either EGFP-2xFYVE or EGFP-2xFYVE(C215S) as described above. The expression of the FYVE-domain interfered with the degradation of the peroxisomal marker protein Fox3p, while the mutated form still allowed pexophagy to occur.

Fig. 5: Screen for PtdIns3P binding proteins involved in pexophagy

The indicated strains were grown on oleic acid media for 16 h and subsequently shifted to glucose-containing medium for the induction of pexophagy. After distinct periods of time, samples were taken, whole cell lysates prepared and subjected to SDS-PAGE and immunoblot analysis. Mitochondrial porin served as an internal control. The degradation of Fox3p (peroxisomal ketoacyl-CoA thiolase) served as a marker for pexophagy, which in wild-type cells leads to the disposal of peroxisomes within 24 hours. The deletion strains were grouped into proteins with (A) FYVE domain, (B) PX domain or (C) proteins with a non-canonical binding motif.

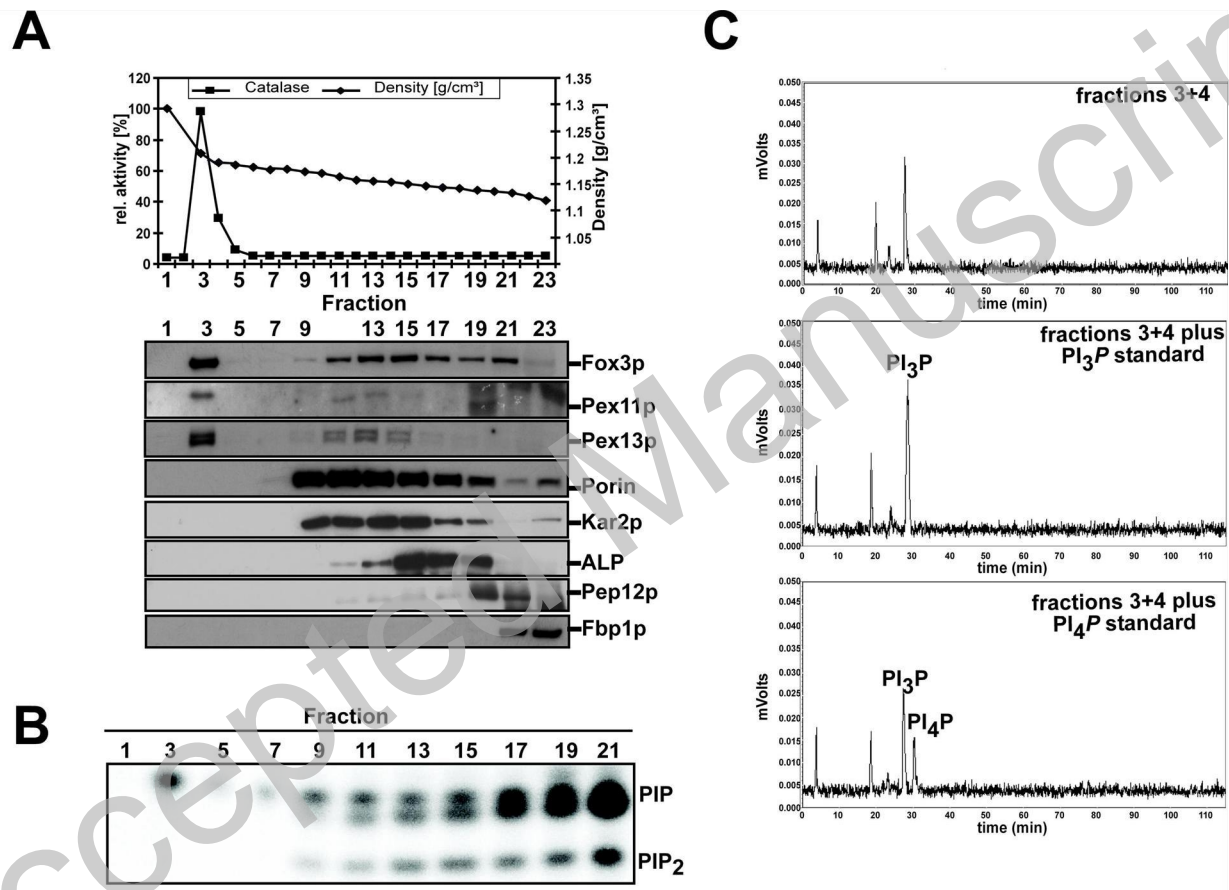


Figure 1

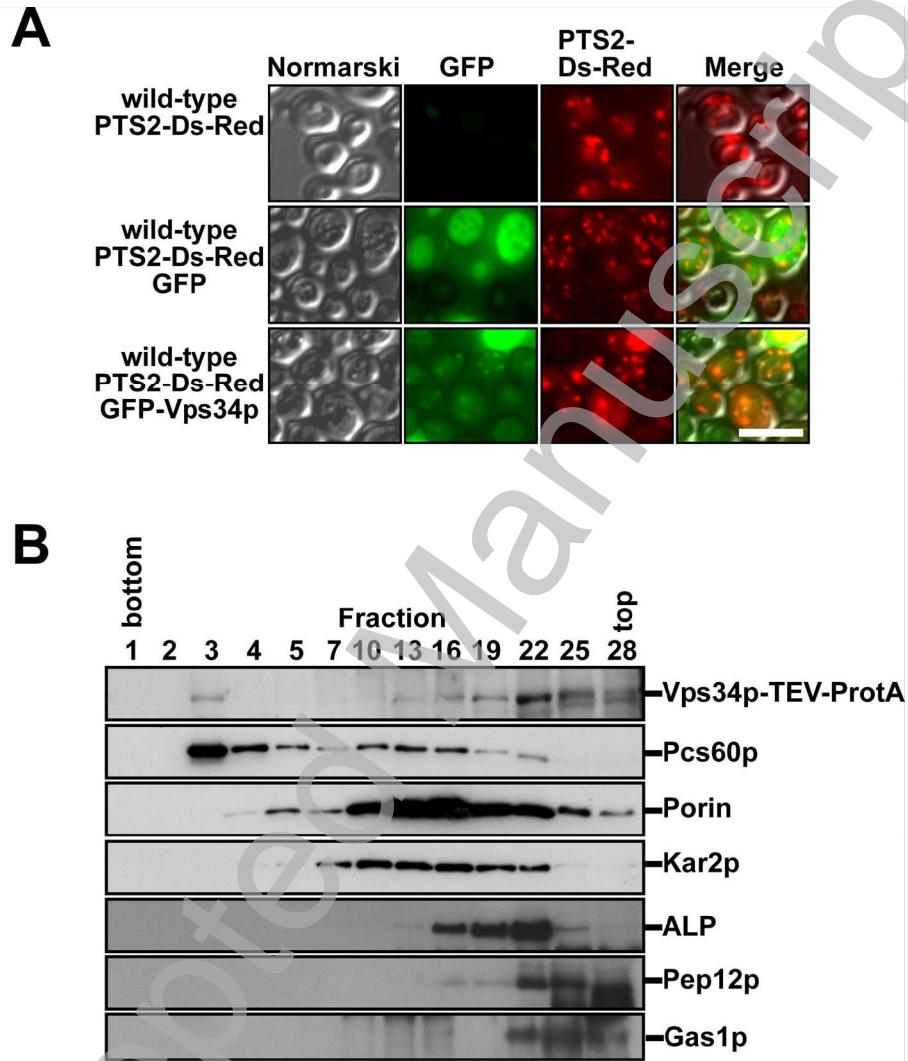


Figure 2

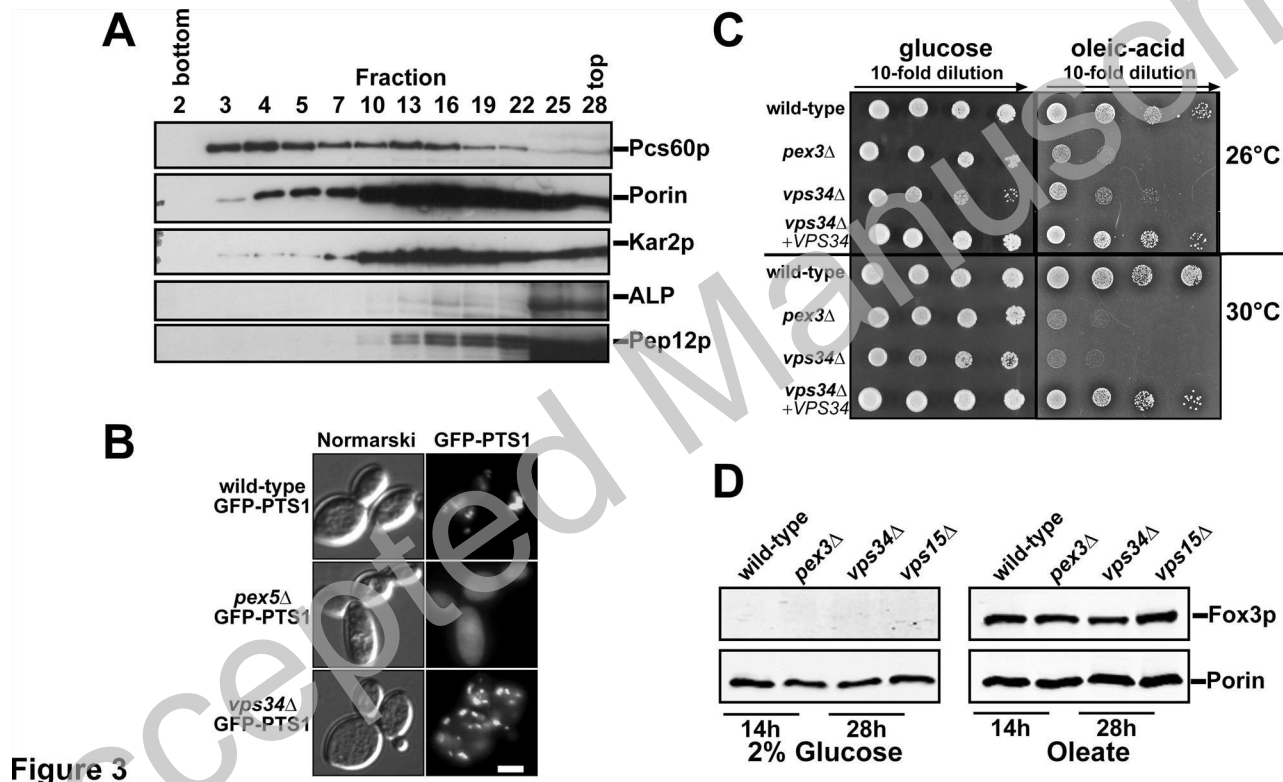
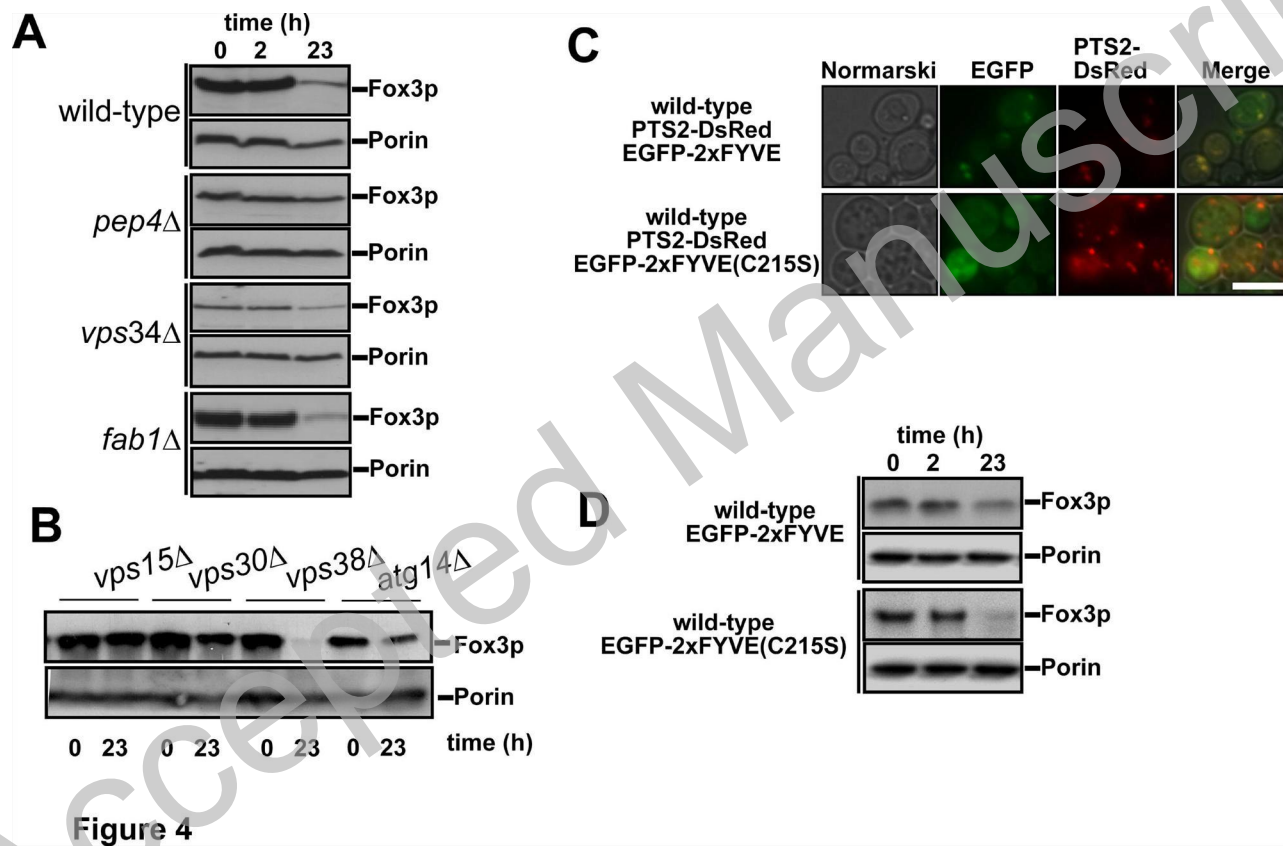


Figure 3



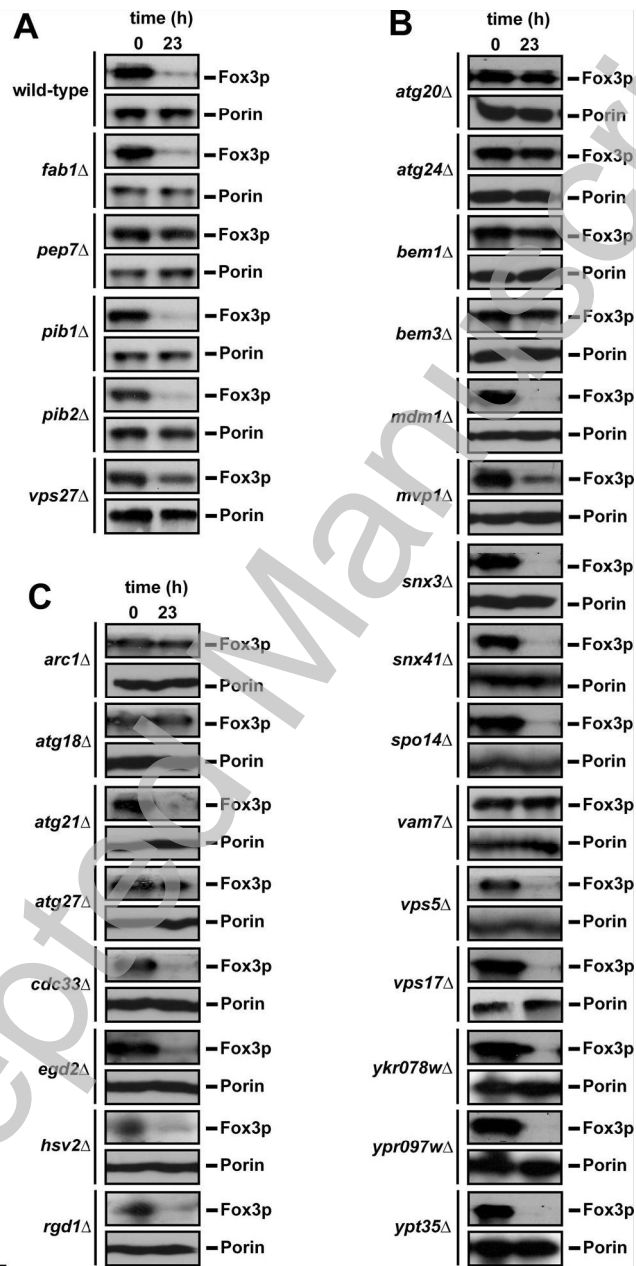


Figure 5