Atg21p is essential for macropexophagy and microautophagy in the yeast *Hansenula polymorpha*☆

Adriana N. Leão-Helder[a,1], Arjen M. Krikken[a], Gerd Gellissen[b,2], Ida J. van der Klei[a], Marten Veenhuis[a], Jan A.K.W. Kiel[a,*]

[a] *Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*
[b] *Rhein Biotech GmbH, Eichsfelder Strasse 11, 40595 Düsseldorf, Germany*

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Abstract ***ATG*** **genes are required for autophagy-related processes that transport proteins/organelles destined for proteolytic degradation to the vacuole. Here, we describe the identification and characterisation of the *Hansenula polymorpha ATG21* gene. Its gene product Hp-Atg21p, fused to eGFP, had a dual location in the cytosol and in peri-vacuolar dots. We demonstrate that Hp-Atg21p is essential for two separate modes of peroxisome degradation, namely glucose-induced macropexophagy and nitrogen limitation-induced microautophagy. In *atg21* cells subjected to macropexophagy conditions, sequestration of peroxisomes tagged for degradation is initiated but fails to complete.**

Keywords: Autophagy; Methylotrophic yeast; Microbody; Pexophagy; Selective peroxisome degradation

1. Introduction

In general, cells respond to new environmental stimuli by adapting their metabolic machinery. In eukaryotes, organelles represent an important portion of the proteins that may be subject to such adaptations. This phenomenon is in particular prominent in case of peroxisomes, which can be induced to proliferate or be degraded in relation to the nutrient composition of the growth medium. In the yeast *Hansenula polymorpha*, proliferation of peroxisomes is induced when methanol is available as sole source of carbon and energy. At these conditions, the organelles harbour the key enzymes of methanol metabolism and thus are essential for growth on this compound. Upon carbon catabolite inactivation (i.e., due to the presence of glucose in the medium), the reverse process occurs and the organelles are degraded by a very selective and efficient process designated macropexophagy (reviewed in [1]). An alternative mode of peroxisome degradation takes place when methanol-grown cells of *H. polymorpha* are subjected to nitrogen (N)-starvation. This condition triggers degradation of portions of the cytoplasm (cytosol and organelles) in a non-selective manner. During this process, the vacuolar membrane is actively engaged in uptake of material that is subsequently degraded. This mode of degradation is known as microautophagy [1,2].

We aim at understanding the process of peroxisome degradation at the molecular level and to this end we have isolated *H. polymorpha* mutants that are disturbed in macropexophagy and analysed the corresponding genes and their encoded proteins. The data gathered so far have demonstrated that macropexophagy in *H. polymorpha* shares protein components with other protein/organelle transport routes to the vacuole that have been described in *Saccharomyces cerevisiae*, including autophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway (reviewed in [1,3,4]) Recently, a unified nomenclature for genes involved in autophagy and related pathways has been adopted and such genes are now referred to as *ATG* genes [5].

In this paper, we report the identification of the *H. polymorpha ATG21* gene. Its gene product, Hp-Atg21p, belongs to a family of WD40 proteins that includes in *S. cerevisiae* Atg18p, Atg21p and Ygr223cp, a protein of unknown function [6–9]. Our current findings, that cells of a *H. polymorpha ATG21* null mutant strain are impaired in both modes of peroxisome degradation, add to the function of Atg21p observed in baker's yeast, where the protein is essential for the Cvt pathway but not for other autophagy-related processes [7,10,11].

2. Materials and methods

2.1. Strains and growth conditions

The *H. polymorpha* strains used in this study are all derivatives of NCYC495 [12] and are listed in Table 1. *H. polymorpha* cells were grown at 37 °C in YPD media (1% yeast extract, 1% peptone, and 1% glucose), selective minimal media (YND) containing 0.67% Yeast Nitrogen Base without amino acids (DIFCO) supplemented with 1% glucose or mineral media (MM; [13]) supplemented with 0.5% glucose

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* Corresponding author. Fax: 31-50-363-21-54.
E-mail address: J.A.K.W.Kiel@biol.rug.nl (J.A.K.W. Kiel).

[1] Present address: Rio de Janeiro State University, Department of Cell Biology and Genetics, Av. São Francisco Xavier, 524, Pavilhão Haroldo Lisboa da Cunha, 2nd floor, 20550-013 Rio de Janeiro, RJ, Brazil.
[2] Present address: MedArtis Pharmaceuticals GmbH, Pauwelstr. 19, 52047 Aachen, Germany.

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Table 1
H. polymorpha strains used in this study

Strain	Genotype and characteristics	Reference
NCYC495	*leu1.1* derivative	[12]
NCYC495	*leu1.1 ura3* derivative	[12]
HF246	NCYC495::(P_{AOX}*eGFP-SKL*)^{1c} *leu1.1*	[20]
Pdd15-REMI	HF246::[pREMI-Z], *leu1.1*, *pdd*, zeo^R	This study
atg21	NCYC495 *atg21::Hp-URA3*, *leu1.1*	This study
atg21+eGFP-SKL	*atg21* ::(P_{AOX}*eGFP-SKL*)^{1c}, *leu1.1*, zeo^R	This study
ATG21-eGFP	NCYC495::*ATG21-eGFP*, *leu1.1*, zeo^R	This study

or 0.5% methanol. Whenever necessary, media were supplemented with 30 μg/ml leucine or 100 μg/ml zeocin.

Escherichia coli DH5α (Gibco-Brl, Gaithesburg, MD) was grown at 37 °C in LB medium (1% trypton, 0.5% yeast extract, and 0.5% NaCl), supplemented with 100 μg/ml ampicillin or 25 μg/ml zeocin as required.

2.2. Miscellaneous DNA techniques

Plasmids used in this study are listed in Table 2. All DNA manipulations were carried out according to standard techniques [14]. *H. polymorpha* cells were transformed by electroporation [15] and yeast chromosomal DNA was isolated as described in [16]. For DNA sequence analysis, the Clone Manager 5 program (Scientific and Educational Software, Durham, USA) was used. BLAST algorithms [17] were used to screen databases at the National Center for Biotechnology Information (Bethesda, MD). The Clustal_X program was used to align protein sequences [18], while the GeneDoc program (available at http://www.psc.edu/biomed/genedoc) was used to display the aligned sequences. The software TREECON for Windows [19] was used for the construction of phylogenetic trees.

Table 2
Plasmids used in this study

Plasmid	Characteristics	Reference
pBluescript II SK$^+$	*E. coli* cloning vector, amp^R	Stratagene, La Jolla, CA
pBSK-URA3	pBluescript SK$^+$ containing the 2.3 kb *H. polymorpha URA3* fragment, amp^R	[24]
pREMI-Z	used for gene-tagging mutagenesis, zeo^R	[20]
pREMI-7	Rescued plasmid of mutant Pdd15-REMI, obtained by digestion of chromosomal DNA with *Eco*RI followed by self-ligation, zeo^R	This study
pKNF38	Rescued plasmid of mutant KNF38 (a RALF mutant disrupted in the *DAK* ORF), obtained by digestion of chromosomal DNA with *Eco*RI followed by self-ligation, zeo^R	[20]
pATG21del	Plasmid containing the cassette for the deletion of the *ATG21* gene, amp^R, *H. polymorpha URA3* gene	This study
pHS6-A	*E. coli*/*H. polymorpha* shuttle vector derived from pBluescript II SK$^+$, amp^R, *S. cerevisiae LEU2* gene, HARS1	[24]
pATG21	pHS6-A containing the *ATG21* gene, amp^R, *S. cerevisiae LEU2* gene, HARS1	This study
pANL29	*H. polymorpha* integrative plasmid containing*AOX* promoter-driven *eGFP-SKL*, zeo^R, amp^R	[24]
pANL31	pBluescript derivative containing the *eGFP* gene without a startcodon, zeo^R, amp^R	[24]
pATG21-eGFP	pANL31 with a 748 bp fragment containing the 3′ end of the *ATG21* gene fused in-frame to the *eGFP* gene, zeo^R, amp^R	This study

2.3. Gene tagging mutagenesis and identification of the Hp-ATG21 gene

The RALF gene tagging method [20] was used to generate mutants in *H. polymorpha* HF246 [20], that were unable to degrade peroxisomes based on the allyl alcohol [21] and alcohol oxidase plate assays [22]. A mutant designated Pdd15-REMI was used for further study. For the identification of the integration site of the tag in the Pdd15-REMI genome, chromosomal DNA was digested with *Eco*RI, self-ligated and transformed to *E. coli*, giving rise to plasmid pREMI-7. Sequence analysis of the genomic regions flanking the vector in pREMI-7 indicated integration of the tag in the common promoter region of 2 ORFs: *H. polymorpha DAK* [23] and an ORF encoding a putative homologue of *S. cerevisiae* Atg21p. The nucleotide sequence of the putative *H. polymorpha ATG21* gene was determined by primer-walking on pREMI-7 and was deposited at GenBank (Accession No. AY383554).

For complementation analysis, a plasmid (pATG21) containing the full-length *ATG21* gene, including its promoter region, was constructed by cloning the 2.8 kb *Eco*RI (blunted)/*Xho*I fragment of pKNF38 between the *Sma*I and *Sal*I sites of pHS6-A [24].

2.4. Construction of an ATG21 null mutant

In the *atg21* mutant, the region comprising nucleotides +328 to +555 of *Hp-ATG21*, corresponding to amino acids 110–185 of the gene product, was replaced by the *URA3* gene (Fig. 1). First, a 1.5 kb *Eco*RV–*Bgl*II fragment of pREMI-7 (containing 327 bp of the 5′ end of *ATG21*) was cloned in *Not*I (blunted) + *Bgl*II-digested pBSK-URA3 [24]. Subsequently, a 1.1 kb *Eco*RI (blunted)–*Pst*I fragment of pREMI-7 (containing 617 bp of the 3′ end of *ATG21*) was cloned into the resulting plasmid that had been digested with *Asp* 718I (blunted) and *Pst*I. From the final plasmid, designated pATG21del, a 3.3 kb *Bam*HI–*Dra*I cassette was obtained and transformed to *H. polymorpha* NCYC495 *leu1.1 ura3*. Southern blotting, using the ECL direct nucleic acid labelling and detection system (Amersham Corp., Arlington Heights, IL), demonstrated proper deletion of *ATG21* (data not

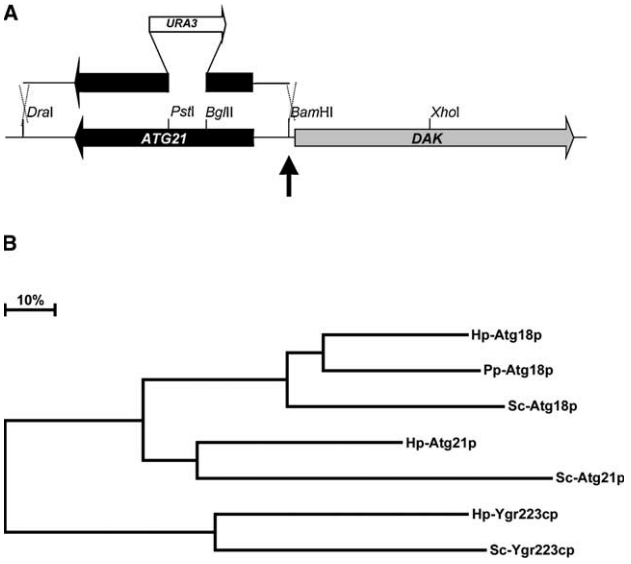

Fig. 1. (A) Schematic representation of the *H. polymorpha* chromosomal region that contains *ATG21* and *DAK*. The strategy for the *ATG21* deletion is also illustrated. Only relevant restriction sites are indicated. The pREMI-Z integration site (indicated by the arrow) is located 51 bp upstream of the *DAK* gene and 240 bp upstream of the putative *ATG21* gene. (B) Evolutionary tree of the putative *H. polymorpha* Atg21p and the other members of a WD40 family identified in *H. polymorpha*, *S. cerevisiae* and *P. pastoris*. The protein encoded by the isolated gene is more closely related to *S. cerevisiae* Atg21p (SwissProt Q02887), than to *H. polymorpha*, *S. cerevisiae* and *P. pastoris* Atg18p (Genbank AY756175 and SwissProt P43601 and Q8X1F5, respectively), while the protein is even less related to *H. polymorpha* and *S. cerevisiae* Ygr223cp (Genbank AY756176 and SwissProt P50079, respectively).

shown). A derivative of the *atg21* strain carrying the peroxisome reporter gene eGFP-SKL (designated *atg21* + eGFP-SKL) was constructed by integrating plasmid pANL29 [24] in its genome.

2.5. Construction of a H. polymorpha *strain synthesising Atg21p-eGFP*

To construct strain ATG21-eGFP, we replaced the genomic copy of the *H. polymorpha ATG21* gene by an *ATG21-eGFP* fusion gene as follows: a 1.1 kb PCR product (containing the 3′ end of *ATG21* lacking its stop codon), obtained using primers *Hin*dIII-ATG21 (5′ AGA AAG CTT GGC GGC GCA AAC TTG G 3′) and ATG21w/oStop (5′ AGA GGA TCC TTC TAT TTT ATA GTG CTT GAG GAG CAC GC 3′), was digested with *Hin*dIII + *Bam*HI and cloned into *Hin*dIII + *Bgl*II-digested pANL31 [24]. The final plasmid, pATG21-eGFP, was linearised with *Bgl*II in the *ATG21* region and transformed to wild-type (WT) *H. polymorpha* NCYC495 *leu 1.1*. Correct integration was confirmed by Southern blotting (data not shown).

2.6. Biochemical and morphological methods

Crude cell extracts were prepared as described [25]. SDS–PAGE and Western blot analysis were performed by established methods. The degradation of peroxisomes in batch cultured cells of *H. polymorpha* was determined as described [22]. Induction of microautophagy by nitrogen limitation was performed as detailed in [2].

Intact cells were prepared for electron microscopy as described previously [26]. Fluorescence microscopy studies were performed using a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany). The vacuolar membrane was stained using the fluorescent lipophilic dye FM 4-64 that was added to yeast cell cultures at 37 °C to a final concentration of 0.4 μM, 1 h before the analysis of cells.

3. Results

3.1. Identification of the Hp-ATG21 gene

A novel *H. polymorpha* mutant deficient in selective peroxisome degradation, designated Pdd15-REMI, was isolated by gene tagging mutagenesis [20]. Sequence analysis of the genomic regions flanking the tag in the mutant genome revealed that the pREMI-Z vector had integrated in the promoter region of two divergently transcribed genes (Fig. 1A). The first gene (*DAK*) encodes the dihydroxyacetone kinase enzyme, which is essential for growth of *H. polymorpha* cells on methanol [23]. Since the capability of the mutant to grow on methanol was not affected, it is unlikely that expression of this gene was significantly decreased. The second gene encoded a protein with a calculated molecular weight of 43 kDa. Primary sequence analyses and BLAST searches indicated that the protein was similar to a family of WD40 repeat proteins, including *S. cerevisiae* Atg18p, Atg21p and Ygr223cp as well as *Pichia pastoris* Atg18p. Of the indicated proteins, Atg18p and Atg21p have been implicated in autophagy-related processes [6–9]. Recently, the nucleotide sequence of the *H. polymorpha* genome was determined [27]. Sequence analysis showed that also the *H. polymorpha* genome encodes three members of this WD40 family. Besides the indicated 43 kDa protein, a predicted protein also can be identified (designated Hp-Atg18p; Genbank Accession No. AY756175) that is highly similar to *P. pastoris* and *S. cerevisiae* Atg18p. Furthermore, a putative homologue of Sc-Ygr223cp is also present (designated Hp-Ygr223cp; Genbank Accession No. AY756176). An alignment of the primary sequences of these proteins is available in the Web version of this manuscript. A phylogenetic tree based on this sequence alignment is depicted in Fig. 1B. Analysis of these data indicates that the isolated protein is most similar to *S. cerevisiae* Atg21p. From this, we concluded that we had isolated the *H. polymorpha ATG21* gene.

3.2. H. polymorpha *atg21 mutant cells are affected in macropexophagy and microautophagy*

Cells of a *H. polymorpha atg21* null mutant grew normally on glucose, glycerol and methanol as respective sole sources of carbon and energy. To investigate whether the isolated gene was involved in peroxisome degradation, we induced macropexophagy by exposing methanol-grown *atg21* cells to excess glucose conditions. As depicted in Fig. 2A, the peroxisomal marker enzyme alcohol oxidase (AO) was not degraded under these conditions in *atg21* cells, relative to WT controls. Also, when *H. polymorpha* cells were exposed to nitrogen-limitation conditions to induce microautophagy, degradation of AO protein was not observed in *atg21* cells within a period of 4 h (Fig. 2B), while in WT controls AO protein was degraded in the same time interval. This indicates that both macropexophagy and microautophagy are blocked in *atg21* cells.

Expression of the full length *ATG21* gene from a self-replicating plasmid restored the ability of the *atg21* mutant cells to degrade AO protein, and thus peroxisomes (data not shown).

3.3. Atg21p acts after initiation of peroxisome sequestration

Macropexophagy in *H. polymorpha* is characteristically initiated by the sequestration of individual peroxisomes tagged for degradation by multiple membrane layers [28]. We carried out microscopic analyses to determine the morphological phenotype of *H. polymorpha atg21* cells. First, cells of strain *atg21*+eGFP-SKL, producing a fluorescent peroxisomal marker protein, were analysed by fluorescence microscopy. During cultivation on methanol, normal fluorescent peroxisomes developed within these cells. Upon a shift of such cells to glucose, eGFP-SKL fluorescence was never observed in the vacuole, indicating that peroxisomes were not taken up by the vacuole (Fig. 3B). Vacuolar uptake of eGFP-SKL fluorescence, and hence peroxisome degradation, was however readily observed in WT controls (Fig. 3A).

We also analysed *atg21* cells during macropexophagy by electron microscopy. Also in these studies, we never observed any morphological characteristics of peroxisomes that had

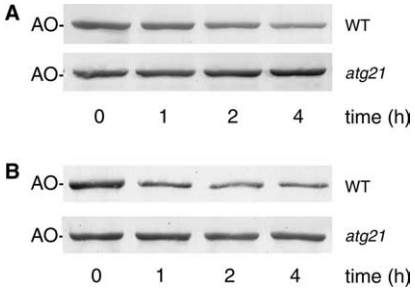

Fig. 2. *H. polymorpha atg21* cells are disturbed in macropexophagy and microautophagy. Western blots were prepared from cell extracts from methanol-grown *H. polymorpha* WT and *atg21* cells subjected to excess glucose conditions to induce macropexophagy (panel A) and from methanol/ammoniumsulfate-grown *H. polymorpha* WT and *atg21* cells shifted to methanol media lacking any nitrogen source (panel B). Samples were taken at the indicated time points. Equal volumes of cultures were loaded per lane. Blots were decorated with polyclonal antibodies against AO protein. At both macropexophagy and microautophagy conditions, the levels of AO protein in WT cells decreased in time indicating degradation of peroxisomes, while in *atg21* cells AO levels remained unchanged.

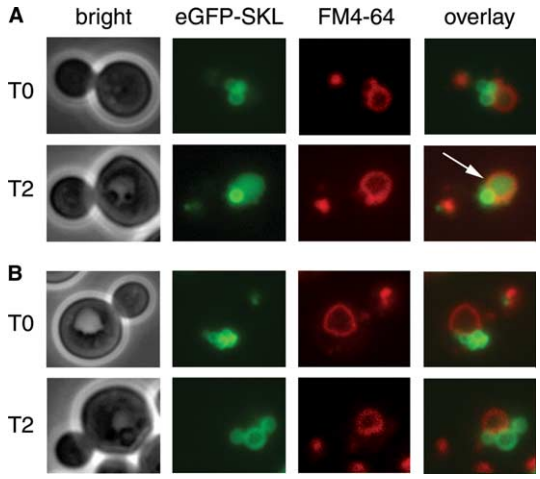

Fig. 3. In *atg21* cells, peroxisomes are not incorporated by the vacuole upon induction of macropexophagy. Fluorescence microscopy studies of *H. polymorpha* WT (panel A) and *atg21* (panel B) cells that synthesise the peroxisomal reporter protein eGFP-SKL (strains HF246 and *atg21*+eGFP-SKL, respectively). Methanol-grown cells were subjected to excess glucose conditions to induce macropexophagy. The vacuolar membrane was stained using the fluorescent dye FM4-64. Two hours after the shift of cells to glucose eGFP-SKL, fluorescence is readily observed in the vacuole of WT cells (arrow), while in cells of the *atg21* mutant the fluorescence remains confined to peroxisomes. T0, methanol-grown cells; T2, 2 h after the addition of glucose.

been taken up by the vacuole (data not shown). Moreover, fully sequestered peroxisomes, the essential pre-stage of macropexophagy, were never observed. However, we regularly detected short stretches of membranes adjacent to peroxisomes that strongly resembled initial stages of organelle sequestration (Fig. 4). Together, these results suggest that in *atg21* cells macropexophagy is blocked at an early step in the sequestration process.

3.4. *Atg21p has a dual location in the cytosol and in peri-vacuolar spots*

To determine the subcellular location of *H. polymorpha* Atg21p, we constructed a strain (ATG21-eGFP) in which the endogenous *ATG21* gene was replaced by an *ATG21-eGFP* fusion gene. Analysis of macropexophagy in ATG21-eGFP cells indicated that the fusion protein is fully functional (data not shown). When cells of this strain were grown on methanol-containing media, Atg21p-eGFP fluorescence was detected in a single – or infrequently few – dots at the vacuolar membrane in conjunction with cytosolic fluorescence (Fig. 5). We have also analysed ATG21-eGFP cells during glucose-induced macropexophagy and nitrogen limitation-induced microautophagy conditions, but we have never observed any changes in the location of Atg21p-eGFP (data not shown).

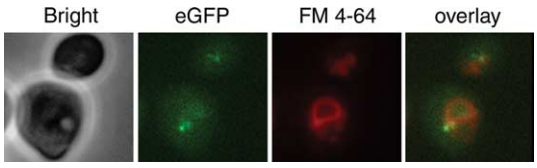

Fig. 5. Atg21p.eGFP is localised to distinct peri-vacuolar dots and in the cytosol. Fluorescence microscopy pictures of methanol-grown *H. polymorpha* ATG21-eGFP cells. The vacuolar membrane was stained using the fluorescent dye FM4-64. Fluorescence is observed in one or few distinct dots at the vacuole in conjunction with fluorescence in the cytosol.

4. Discussion

Here, we report the identification and characterisation of the *H. polymorpha ATG21* gene. Its protein product, Atg21p, belongs to a WD40 repeat protein family consisting of Atg18p, Atg21p and Ygr223cp. In these proteins, the WD40 repeats are predicted to fold into a seven-bladed propellor [29]. In baker's yeast, Atg21p is essential for the Cvt pathway, while N-limitation induced autophagy-like processes are only partially disturbed [8,10,11]. Our current data of *H. polymorpha* therefore add a role for Atg21p in macropexophagy, while the function of the protein in microautophagy is much more pronounced, relative to baker's yeast [10]. Remarkably, cells of *S. cerevisiae* and *P. pastoris* deleted for the related protein

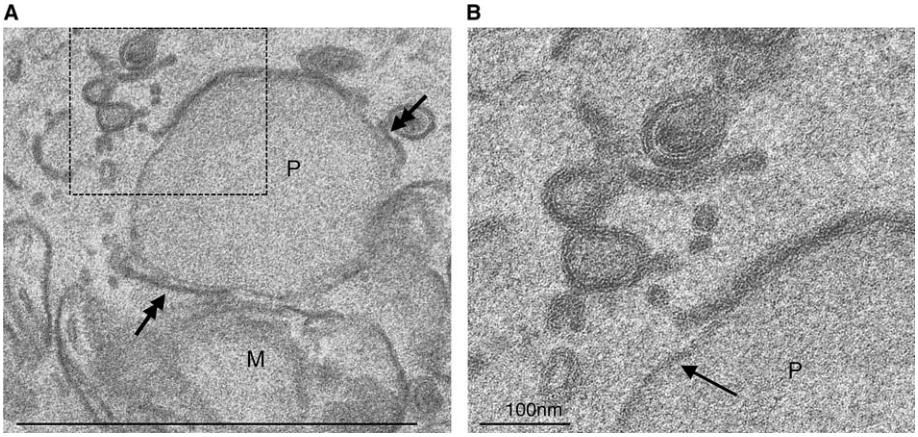

Fig. 4. Peroxisomes become only partially sequestered upon induction of macropexophagy in *atg21* cells. Morphology of methanol-grown *atg21* cells that were exposed to glucose-excess conditions for 30 min. Cells were fixed with $KMnO_4$. Panel A. Section of a cell showing the characteristic stretches of sequestering membrane that adhere to the peroxisomes (double arrowheads), but fail to sequester the organelles. The other membrane material observed in the cytosol is likely of same nature but not connected to the organelle. Panel B. Enlargement of the indicated region in panel A. The arrow indicates a region of the peroxisomal membrane that was not sequestered. *M*, mitochondrion; *P*, peroxisome. The bar in panel A represents 1 μm.

Atg18p are fully deficient in all autophagy-related pathways, including pexophagy [7,9]. The third member of this family, Sc-Ygr223cp, appears not to be involved in autophagy-related processes at all [10,11]. Recently, it was demonstrated that baker's yeast Atg18p, Atg21p and Ygr223cp are actually phosphoinositide binding proteins [10,29]. Thus, we presume that in *S. cerevisiae* Atg18p and Atg21p have partly redundant functions, a feature that apparently is not conserved in *H. polymorpha*.

Morphological analysis of *H. polymorpha atg21* cells subjected to macropexophagy conditions suggested that the peroxisome sequestration process was not completed and fusion with the vacuole did not take place. This suggests that in *H. polymorpha atg21* cells, the processes that signal and initiate sequestration of tagged organelles are not affected. Rather, *atg21* cells appear to be blocked in the development of the sequestering membrane. The frequently observed loose association of the sequestering membranes with the organelles might imply that the function of the machinery that controls the close connection of these membranes with the peroxisomal membrane is affected. In both *S. cerevisiae atg18* and *atg21* cells, sequestration of the Cvt cargo aminopeptidase I is absent or not completed [9–11], clearly resembling the incomplete sequestration of peroxisomes observed in *H. polymorpha atg21* cells. Also, in a *P. pastoris atg18* mutant membranes sequestering peroxisomes were reported to be absent during macropexophagy [9].

In methanol-grown *H. polymorpha* cells, Atg21p-eGFP is located in single (or few) dot(s) at the vacuole and in the cytosol. Furthermore, our data suggest that the location of Atg21p-eGFP remains unchanged during macropexophagy and microautophagy. Similar locations have been observed for tagged versions of *S. cerevisiae* Atg18p [6,9], Atg21p [7,10,11] and Ygr223cp [10], and in *P. pastoris* for overproduced HA-Atg18p [9]. Also in *S. cerevisiae*, induction of autophagy did not result in a changed location for these WD40 repeat proteins. This might suggest that these proteins are actually present on identical structures, but are not incorporated into Cvt vesicles, autophagosomes or sequestering peroxisomes, a notion that requires further investigation.

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