RESEARCH ARTICLE

The role of *Hansenula polymorpha MIG1* homologues in catabolite repression and pexophagy

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Abstract

In the methanol-utilizing yeast *Hansenula polymorpha*, glucose and ethanol trigger the repression of peroxisomal enzymes at the transcriptional level, and rapid and selective degradation of methanol-induced peroxisomes by means of a process termed pexophagy. In this report we demonstrate that deficiency in the putative *H. polymorpha* homologues of transcriptional repressors Mig1 (*HpMig1* and *HpMig2*), as well as HpTup1, partially and differentially affects the repression of peroxisomal alcohol oxidase by sugars and ethanol. As reported earlier, deficiency in HpTup1 leads to impairment of glucose- or ethanol-induced macropexophagy. In *H. polymorpha mig1mig2* double-deletion cells, macropexophagy was also substantially impaired, whereas micropexophagy became a dominant mode of autophagic degradation. Our findings suggest that homologues of the elements of the *Saccharomyces cerevisiae* main repression pathway have pleiotropic functions in *H. polymorpha*.

Introduction

It is well established that intact peroxisomes are indispensable for methylotrophic growth in yeasts. These organelles harbour key enzymes that catalyze the first steps of methanol utilization [as alcohol oxidase (AO), catalase, dihydroxyacetone synthase], and provide compartmentalization of toxic methanol catabolites, formaldehyde and hydrogen peroxide (Veenhuis *et al*., 1983b; van der Klei *et al*., 2006). Peroxisomes, however, are redundant for growth on rich carbon sources such as hexoses and disaccharides. When methanol-grown cells are shifted to sugar carbon substrates or ethanol, peroxisomes are rapidly and selectively degraded in vacuoles (Veenhuis *et al*., 1983a), whereas genes of peroxisomal enzymes are subjected to tight transcriptional repression (Roggenkamp *et al*., 1984). Such coordinated genetically programmed catabolite regulation ensures a fast and efficient cellular response to environmental changes.

It was originally assumed that the molecular pathway of glucose catabolite repression in methylotrophic yeasts may mimic that of the conventional yeast *Saccharomyces cerevisiae* (the so-called Snf1-Mig1 pathway). Its main components, the lack of which abolishes repression signalling, are hexokinase II subunit, Snf1 protein kinase, and the Mig1 repressor that tethers or facilitates the binding of the Tup1-Ssn6 general repressor to promoters of repressible genes in the presence of glucose (Gancedo, 1998; Papamichos-Chronakis *et al*., 2004). Several observations support this hypothesis. First, Mig1-Ssn6-Tup1 and the Snf1 complexes are highly conserved among different yeasts (Gancedo, 1998). Second, promoters of *H. polymorpha* alcohol oxidase (Pereira & Hollenberg, 1996) and maltase (Alamae *et al*., 2003) genes were demonstrated to be repressed by glucose in *S. cerevisiae*, suggesting that they are targets of the host's repression pathway. Recently, hexose phosphorylation activity was shown to be essential for repression signalling in *H. polymorpha*: hexokinase mutant was insensitive to fructose (but not glucose) repression, whereas in double hexo- and glucokinase mutant, glucose repression was also abrogated (Kramarenko *et al*., 2000). The above findings suggested

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that glucose-repressible genes of C-1 metabolism in *H. polymorpha* may be the target of Mig1/(Tup1-Ssn6)-mediated transcriptional repression, analogous to the case of baker's yeast. However, Oliveira *et al.* (2003) reported that HpTup1 is not essential for glucose repression of peroxisomal enzymes in *H. polymorpha*.

Adding to the emerging complexity of repression pathway in this yeast, we recently identified a hexose transporter homologue, HpGcr1, which is most probably involved in the first stages of glucose (but not of sucrose or ethanol) signalling for repression, but may also interfere with high-affinity glucose transport (Stasyk *et al.*, 2004). Its close homologues in *S. cerevisiae*, the putative glucose sensors Snf3 and Rgt2, were not implicated in the repression mechanism, but instead differentially regulate the induction of functional hexose transporters in response to extracellular glucose (Johnston & Kim, 2005). It has to be emphasized that, in *H. polymorpha* and other methylotrophs, disaccharides and ethanol are potent repressors of gene expression involved in C-1 metabolism (Sibirny *et al.*, 1988; Stasyk *et al.*, 2004). However, nothing is known of the specific molecular components of ethanol- or disaccharide-triggered repression, whether different carbon source-dependent pathways physically converge, and, if so, at what stage they do so.

With regard to pexophagy, significant progress has been achieved in recent years in understanding its molecular mechanisms and regulation, with *H. polymorpha* being a very productive model organism, as detailed in recent reviews (Dunn *et al.*, 2005; Monastyrska & Klionsky, 2006; Sakai *et al.*, 2006).

Two morphologically distinguishable pexophagy mechanisms have been observed in *H. polymorpha*: selective macropexophagy-triggered by a shift in carbon source (i.e. from methanol to glucose or ethanol) (Veenhuis *et al.*, 1983a; Tuttle *et al.*, 1993), or cold shock (Komduur *et al.*, 2004); and nonselective micropexophagy triggered upon nitrogen starvation, leading to bulk turnover by general autophagy (Monastyrska *et al.*, 2002). Most *ATG* (AuTopHaGy-related) genes involved in pexophagy identified so far in *H. polymorpha* and other yeasts are conserved among eukaryotes and also appear to affect general autophagy. Those essential only for pexophagy are in many cases organism-specific (Meijer *et al.*, 2007). The latter include *H. polymorpha ATG11* and *ATG25* (Monastyrska *et al.*, 2005), *ATG26* (our unpublished results), and *TUP1* (Leao-Helder *et al.*, 2004). HpTup1 is a first representative of transcriptional repressors involved in pexophagy. As macropexophagy, in contrast to nitrogen starvation-induced microautophagy, is insensitive to cycloheximide (CHX) (i.e. independent of protein synthesis *de novo*) (Monastyrska *et al.*, 2005), the function of HpTup1 in pexophagy has been suggested to be indirect (Leao-Helder *et al.*, 2004).

Knowledge of the molecular mechanisms of catabolite regulation in *H. polymorpha* is of basic and practical interest. Mutants with altered catabolite regulation of the AO gene promoter may be promising hosts for the expression of recombinant proteins in methanol-free media (Krasovska *et al.*, 2007), and protein products can be targetted to peroxisomes applying this organelle as a storage vesicle (van Dijk *et al.*, 2000). The availability of the full *H. polymorpha* genome sequence (Ramezani-Rad *et al.*, 2003) made it possible to search for orthologues of *S. cerevisiae* genes involved in catabolite repression and to study their function. In this report we analyse the possible role of two *H. polymorpha* Mig1 homologues (designated HpMig1 and HpMig2) in the processes of catabolite repression and pexophagy.

Materials and methods

Microorganisms and growth conditions

The following *H. polymorpha* strains were used in this study: NCYC495 *leu1-1* (Gleeson & Sudbery, 1988), Δ*mig1* deletion mutant, Δ*mig2* deletion mutant, Δ*mig1*Δ*mig2* double-deletion mutant (all this study), Δ*tup1 leu1-1* (Leao-Helder *et al.*, 2004), Δ*gcr1* (Stasyk *et al.*, 2004).

Hansenula polymorpha cells were grown at 37 °C in rich YPD medium (1% peptone, 2% yeast extract, 1% glucose) or minimal media containing 0.67% yeast nitrogen base (YNB, Difco, Detroit, MI) supplemented with 1% glucose (YND), 1% sucrose (YNS), 1% ethanol (YNE), or 1% methanol (YNM), unless stated otherwise. For auxotrophic strains, leucine ($40\,mg\,L^{-1}$) was added. For the selection of yeast transformants via geneticine (G418) resistance, $0.4\,g\,L^{-1}$ of G418 was added to YPD plates.

The *Escherichia coli* DH5α strain was used as a host for propagation of plasmids and grown at 37 °C in Luria–Bertani (LB) medium as described in Sambrook *et al.* (1989).

Molecular-biology techniques

Standard cloning and DNA manipulation techniques were applied (Sambrook *et al.*, 1989). Genomic DNA of *H. polymorpha* was isolated using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Restriction endonucleases and DNA ligase (Fermentas, Vilnius, Lithuania) were used according to the manufacturer's specifications. PCR-amplification of the fragments of interest was performed with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) according to the manufacturer's specification. The primers used in this study are listed in Table 1. The transformation of *H. polymorpha* by electroporation was carried out as described previously (Faber *et al.*, 1994).

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Table 1. Primers used in this study

Gene specificity	Name of primer	Sequence
HpMIG1	OL54	5′-TGTGGATCCTTTTTTGGCGTGGATGT-3′
HpMIG1	OL55	5′-GGTTCTAGATCACTCAGAAAACATGG-3′
HpMIG1	OL57	5′-TGGAAGCTTAGAAAGACCTGCTTGCTG-3′
HpMIG1	Mi12Nf	5′-CCCAAGCTTGGTGTAGTTGTCGGAG-3′
HpMIG1	Mi12Nr	5′-ATAAGAATGCGGCCGCCTACACTGTAGCTACG-3′
HpMIG1	Mi12Cf	5′-CGAGACCTGCATACATG-3′
HpMIG1	Mi12Cr	5′-CCGGAATTCGCATCCCAGAGTACTC-3′
HpMIG2	OL91	5′-AAACTAAAACAGGGATCG-3′
HpMIG2	OL92	5′-AAAATACGACTGCAGCGA-3′
HpMIG2	OL153	5′-TGTGGATCCAAATTTTCACACCTCAGG-3′
HpMIG2	OL154	5′-CCGATATATCTAGAGACGTGCTTCCAT-3′
HpMIG2	OL155	5′-TCCATAGAAAGCTTCTGAACAGCCCCGAAGA-3′
HpMIG2	OL156	5′-GGAAGCTTGAGCTCGAAATATTTCCTTCTTTGC-3′
HpLEU2	OL143	5′-GCACAATGTTCTTACTCAT-3′
ScLEU2	CK15	5′-TGTAATTGTTGGGATTCC-3′

Protein sequence analyses

For analysis of DNA and amino acid sequences, MACVECTOR software (IBI, New Haven, CT) was used. Sequence alignments and phylogenetic analysis were performed using the CLUSTALW version 1.6 algorithm (Corpet, 1988). The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD; http://www.ncbi.nlm.nih.gov/BLAST/) was used to search for amino acid sequence similarities. For pattern and profile search, the PROSCAN program at http://npsa-pbil.ibcp.fr/cgibin/pattern_prosite.pl was used (Combet *et al.*, 2000).

Construction of *HpMIG1* and *HpMIG2* deletion strains

A *HpMIG1* (GenBank accession number EF523343) deletion cassette was constructed as follows: a 0.93-kb fragment containing sequences of the 3′-flanking region of *HpMIG1* was amplified by PCR using genomic DNA of *H. polymorpha* as a template and the primers Mi12Cf and Mi12Cr carrying restriction sites for EcoRI and BamHI (Table 1). The 3′-flanking fragment was inserted into the EcoRI/BamHI-digested plasmid pBSKT (Lombo *et al.*, 1997). Next, a 0.99-kb fragment consisting of the 5′-flanking region of *HpMIG1* was amplified by PCR with the primers Mi12Nf and Mi12Nr (Table 1) carrying restriction sites for HindIII and NotI, respectively. The 5′-flanking PCR fragment of *HpMIG1* and BamHI/NotI-digested *HpLEU2* fragment were cloned into the BamHI/NotI-digested plasmid pBSKT-*3′MIG1*, thus generating a deletion cassette for *HpMIG1* (plasmid p19-mig12::*HpLEU2*). The derivative plasmid pOH7 was constructed by replacing the PstI fragment of *HpLEU2* on p19-mig12::*HpLEU2* with the PstI fragment harbouring the *APH* gene expressed under the constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase from plasmid pGLG578 (Sohn *et al.*, 1999) (Fig. 1).

The Δ*mig1* and Δ*mig1 leu1-1* strains were isolated by transforming recipient wild-type strain NCYC495 *leu1-1* with deletion cassettes for *HpMIG1* from p19-mig12::*HpLEU2* (EcoRI–HindIII fragment comprising *HpLEU2* flanked by *HpMIG1* 5′- and 3′-sequences) and pOH7 (Eco32I/PaeI fragment of pOH7 comprising *APH* flanked by the same *HpMIG1* sequences), thereby replacing the region of *HpMIG1* either with *HpLEU2* or with *APH*. Prototrophic or G418-resistant transformants were isolated and analysed as detailed below.

A *HpMIG2* (GenBank accession number EF523344) deletion cassette was constructed as follows: a 1.97-kb fragment containing sequences of the 3′-flanking region of *HpMIG2* was amplified by PCR using genomic DNA of *H. polymorpha* NCYC495 *leu1-1* as a template and the primers OL155 and OL156 carrying restriction sites for HindIII (Table 1, Fig. 1). The 3′-flanking fragment was inserted into the HindIII-digested plasmid pYT1 (Tan *et al.*, 1995), carrying the *ScLEU2* gene as a selection marker, to create pYT1-*3′MIG2*. Next, a 1.39-kb fragment consisting of the 5′-flanking region of *HpMIG2* was amplified by PCR with the primers OL153 and OL154 carrying restriction sites for BamHI and XbaI, respectively. The 5′-flanking PCR fragment of *HpMIG2* was cloned into the BamHI/XbaI-digested plasmid pYT1-*3′MIG2*, to create pOH8, generating a deletion cassette for *HpMIG2*.

The plasmid pOH8 was used as a template for PCR with primers OL153 and OL156, producing a 5.56-kb fragment comprising *ScLEU2* flanked by *MIG2* 5′- and 3′-sequences. The deletion cassette was transformed into NCYC495*leu1-1*, and prototrophic transformants selected.

Total genomic DNA isolated from potential Δ*mig1* knock-out strains (p19-mig12::*HpLEU2* and pOH7 transformants) was used as a template for PCR and Southern blot analyses. Primer pairs OL54/OL55, OL54/OL57 and OL54/OL143 (Table 1, Fig. 1) were used to determine the presence

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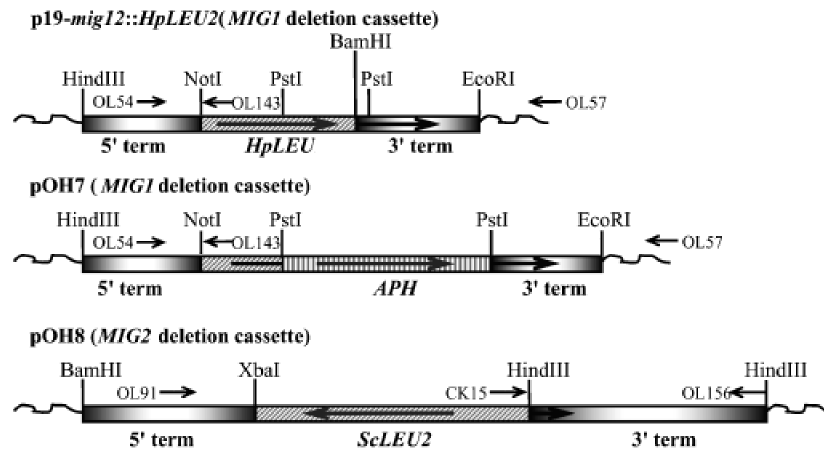

Fig. 1. Scheme of construction of *Hansenula polymorpha* Δ*mig1* and Δ*mig2* deletion strains. The *HpMIG1*, *HpMIG2*, *HpLEU2*, *ScLEU2* and *APH* genes and directions of transcription are indicated by arrows in plasmids p19-*mig12*::*HpLEU2*, pOH7 and pOH8. p19-*mig12*::*HpLEU2* consists of a 943-bp fragment containing the 5′ uncoding region of the *HpMIG1* gene (HindIII–NotI section), and a 991-bp fragment carrying the 3′ uncoding region and short terminal part of the *HpMIG1* ORF (BamHI-EcoRI section). pOH7 consists of a 943-bp fragment containing the 5′ uncoding region of the *HpMIG1* gene (HindIII-NotI section), and a 897-bp fragment carrying the 3′ uncoding region and short terminal part of the *HpMIG1 ORF* (PstI–EcoRI section). pOH8 consists of a 1.39-kb fragment containing the 5′ uncoding region of the *HpMIG2* gene (BamHI–XbaI section), and a 1.9-kb fragment carrying the 3′ uncoding region and short terminal part of the *HpMIG2 ORF* (HindIII–HindIII section). Genomic DNA of the *H. polymorpha* that flank deletion cassettes upon integration into genome is represented by wavy line. Names and sequence position of the primers used in plasmid construction and PCR analysis of deletion mutants are indicated.

of corresponding knock-out cassettes in Δ*mig1* and Δ*mig1 leu1-1* genomic DNA. PCR analysis (data not shown) indicated the presence of expected bands in the tested strains: a 0.45-kb band generated by OL54/OL55, a 2.24-kb band (OL54/OL57), and the absence of the band (OL54/OL143) in the control strain NCYC495*leu1-1*. The 2.91-kb band (for Δ*mig1*) and 3.9-kb band (for Δ*mig1leu1-1*) generated by OL54/OL57 and the 0.5-kb band generated by OL54/OL143 were present in knock-out strains. The pair of primers OL54/OL55 did not generate a band in the Δ*mig1* and Δ*mig1 leu1-1*strains.

Primer pairs OL91/OL92 and CK15/OL156 (Table 1, Fig. 1) were used for PCR analysis of the Δ*mig2* strain. The 1.2-kb band generated by OL91/OL92 from genomic DNA of NCYC495*leu1-1* was absent in the deletion strain. The pair of primers CK15/OL156 was used for indication of a correctly targetted chromosomal integration of *mig2::HpLEU2* fragment. The 2.5-kb band was obtained with PCR for Δ*mig2* and was absent in the control strain NCYC495 *leu1-1*. Proper integration of the single *HpMIG1* and *HpMIG2* disruption cassettes was confirmed by Southern blot analysis (data not shown).

The identified Δ*mig1* was used in all biochemical and physiological experiments in this study. Δ*mig1 leu1-1* was used for construction of the Δ*mig1*Δ*mig2* double-deletion mutant. The Δ*migl leu1-1* and Δ*mig2* strains were crossed and diploid hybrids were selected on YNS medium supplemented with G418 ($1.0\,g\,L^{-1}$) without leucine. After sporulation, the Δ*mig1*Δ*mig2* double-deletion mutant was selected among spore progeny on the same medium, and confirmed for the presence of *HpMIG1* and *HpMIG2* deletion cassettes as detailed above for single-deletion mutants. Mating and sporulation techniques were performed using established procedures (Gleeson & Sudbery, 1988).

Biochemical and ultrastructural analyses

Preparation of crude cell extracts, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analyses and preparation of cells for electron microscopy and immunocytochemistry were carried out as described in Waterham *et al.* (1994).

Results

Identification of *H. polymorpha* homologues of the *S. cerevisiae* Mig1 transcriptional repressor

A search in the *H. polymorpha* genome database (Ramezani-Rad *et al.*, 2003) revealed the presence of two putative homologues of *S. cerevisiae* Mig1 (ScMig1) C2H2 zinc-finger (ZnF) transcriptional repressor (Nehlin & Ronne, 1990). Similar results were obtained after probing the genome database with full-length ScMig1, or its N-terminal ZnF DNA-binding domain, which is highly conserved in all yeast Mig1 homologues (Ostling *et al.*, 1996; Cassart *et al.*, 1997; Zaragoza *et al.*, 2000; Carmona *et al.*, 2002), or the full-length sequence of the related *S. cerevisiae* Mig2 protein (Lutfiyya & Johnston, 1996). As expected, the two identified *H. polymorpha* Mig1 homologues, designated as *HpMig1* and *HpMig2*, exhibit limited overall homology to other yeast Mig proteins:

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for instance, 26% identity and 41% similarity to ScMig1; 32% identity and 43% similarity to *Candida albicans* Mig1 (Zaragoza *et al.*, 2000); and 34% identity and 45% similarity to *Schwanniomyces occidentalis* Mig1 (Carmona *et al.*, 2002). However, in the region of N-terminal ZnF domains, the similarity is high: 80% and 76% identity to ScMig1 for HpMig1 and HpMig2, respectively (Fig. 2a). Other predicted *H. polymorpha* proteins from the genome database exhibit $< 50\%$ identity in the ZnF region to ScMig1.

HpMig1 and HpMig2 differ in length (480 and 402 amino acid residues, respectively) and are 33% identical and 44% similar to each other. Both *H. polymorpha* proteins exhibit $< 69\%$ identity in their ZnF domains to ScMig2, and $< 66\%$ to the related *S. cerevisiae* Yer028c (Mig3) (Lutfiyya *et al.*, 1998). However, the DNA-binding domains of both *H. polymorpha* Mig proteins are the most similar ($> 85\%$ identity) to the corresponding regions of Mig-like CreA repressors from filamentous fungi (Ronne, 1995).

Despite the low sequence conservation aside the ZnF region, yeast Mig homologues possess other semi-conserved regions (Cassart *et al.*, 1997), present also in HpMig1 and HpMig2 (Fig. 2b and c), i.e. the so-called C-terminal 'effector domain' (Ostling *et al.*, 1996). In addition, the *H. polymorpha* proteins harbour an amino acid region highly similar to fungal CreA repressors that is absent in many yeast Mig proteins (Fig. 2d). They also possess consensus phosphorylation sites for cAMP-dependent protein kinase, and Snf1 protein kinase (not shown), and therefore may potentially be the subject of corresponding regulation (Treitel *et al.*, 1998). Taken together, our analyses suggest that the identified HpMig1 and HpMig2 most probably represent potential ScMig1 orthologues of this methylotrophic yeast. The corresponding sequences were deposited in GenBank under accession numbers EF523343 (*HpMIG1*), and EF523344 (*HpMIG2*).

Isolation and physiological characterization of the *H. polymorpha* Δ*mig1*, Δ*mig2* and Δ*mig1*Δ*mig2* double-mutant strains

To analyse the physiological significance of the HpMig1 and HpMig2 repressors, we constructed *H. polymorpha* deletion strains in the corresponding genes by gene replacement (Fig. 1; for details see 'Materials and methods'). Several Δ*mig1* and Δ*mig2* null mutants were isolated and all were identical with respect to their phenotype (see below). Because

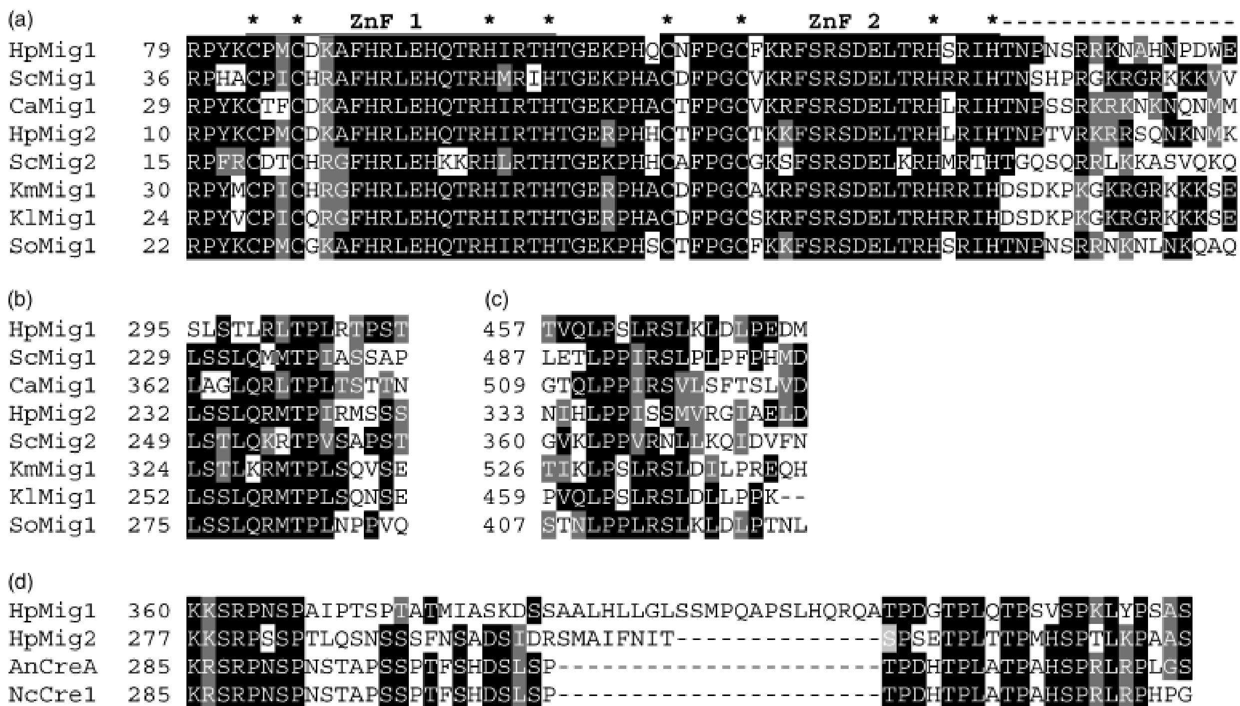

Fig. 2. Multiple alignment of conserved regions of HpMig1 and HpMig2 proteins with yeast Mig1 and fungal *CreA* homologues. (a) N-terminal zinc-finger region. Two zinc-finger domains (ZnF) are overlined. Cysteine and histidine residues involved in ZnF domains are indicated with asterisks. Adjacent to ZnF basic region is overlined. (b) Highly conserved region of yeast Mig proteins. (c) Putative C-terminal effector domain. (d) Highly conserved region between *H. polymorpha* Mig and fungal CreA repressors. Species abbreviations are: Hp, *Hansenula polymorpha*; Sc, *Saccharomyces cerevisiae*; Ca, *Candida albicans*; Km, *Kluyveromyces marxianus*; Kl, *Kluyveromyces lactis*; So, *Schwanniomyces occidentalis*; An, *Aspergillus nidulans*; Nc, *Neurospora crassa*. GenPept accession numbers for proteins used in the alignments: ScMig1 – P27705; ScMig2 – P53035; CaMig1 – Q9Y7G2; KmMig1 – P52288; KlMig1 – P50898; SoMig1 – CAD10675; AnCreA – AAR02858; NcCre1 – O59958.

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HpMig1 and HpMig2 may have a redundant function in transcriptional regulation as observed in *S. cerevisiae* (Lutfiyya *et al.*, 1998), a double-deletion Δ*mig1*Δ*mig2* mutant strain was isolated from spore progeny of the diploid, resulting from crossing Δ*mig1* and Δ*mig2* null mutants.

We observed that Δ*mig1* and Δ*mig2* mutants did not differ from the wild-type strain in growth on solid media supplemented with various carbon sources, namely glucose, sucrose (glycolytic substrates), ethanol and methanol, whereas the double-deletion mutant exhibited retarded growth on sugar substrates (Fig. 3a). The mutants behaved similarly on plates with low sugar concentrations (0.1% w/v) (not shown). However, Δ*mig1* and Δ*mig1*Δ*mig2* mutants could grow on methanol plates in the presence of 2-deoxyglucose, suggesting an impairment of glucose repression. The *H. polymorpha* Δ*tup1* mutant, which is not disturbed for glucose repression (Oliveira *et al.*, 2003), was used as a control.

Detailed analysis of growth kinetics in liquid cultures confirmed that *mig* mutants exhibit a wild-type growth rate on methanol and ethanol. When grown on sugar substrates, the lag phase in Δ*mig1* and double Δ*mig1*Δ*mig2* mutants was extended, but growth was not affected in the exponential phase (Fig. 3b). This observation suggests that HpMig1 and HpMig2 may be involved in a transient transcriptional response upon glucose adaptation.

Assessment of the role of HpMig1, HpMig2 and HpTup1 in catabolite repression

To gain further insight into HpMig1, HpMig2 and HpTup1 function in repression triggered by rich carbon sources, we assayed AO protein levels in the mutants. A very moderate impairment of AO repression was observed for Δ*mig1*, Δ*mig1*Δ*mig2* and Δ*tup1* mutants incubated with low (0.1%) or high (1%) concentrations of glucose (Fig. 4). Pre-

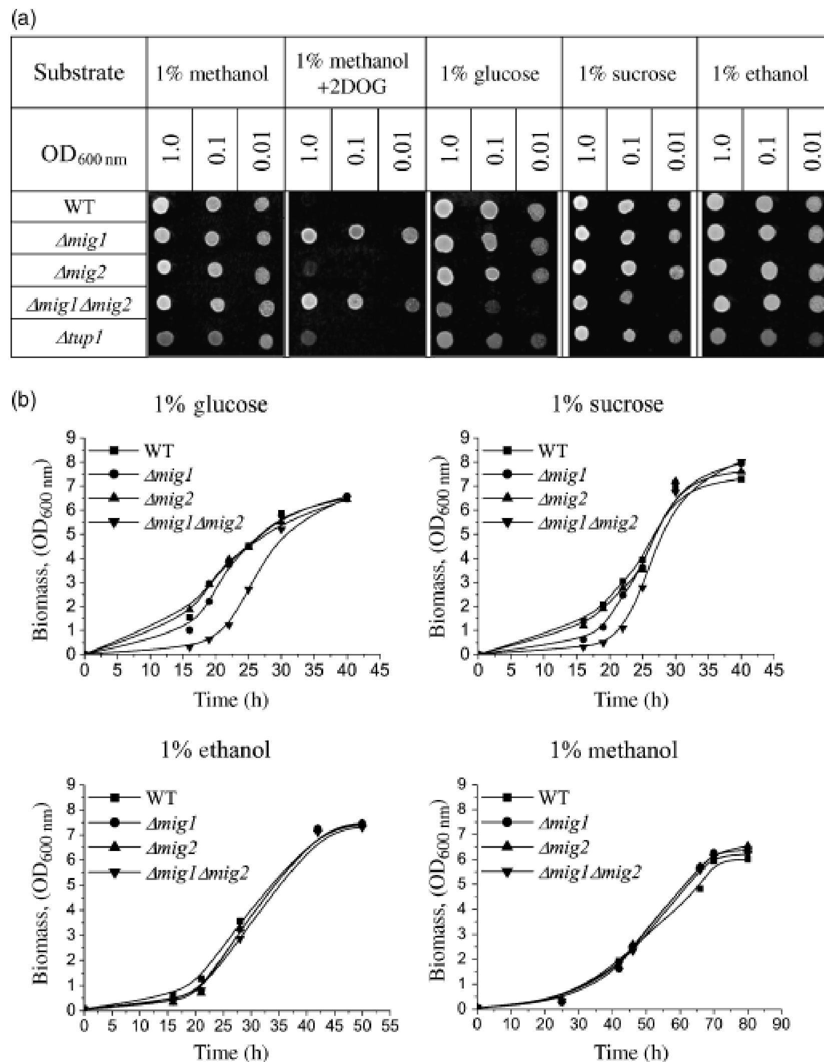

Fig. 3. Growth properties of *Hansenula polymorpha mig* mutants on various carbon sources. (a) Equal volumes of YPS pre-grown cell suspensions were loaded in three dilutions on agarized media. Carbon substrate abbreviations are: Mth, methanol; Glc, glucose; 2-DOG, 2-deoxyglucose; Sucr, sucrose; Eth, ethanol. Cells were incubated for 1 day on sugar substrates, 2 days on methanol or ethanol, and 3 days on 2-DOG-containing medium. (b) Kinetics of growth in the liquid culture. Cells of the indicated *H. polymorpha* strains were pregrown on rich YPS medium and shifted to the mineral media with different carbon sources. $OD_{600\,nm}$ was measured at the indicated time points after the shift.

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viously described by us Δ*gcr1* mutant strongly affected in glucose repression and constitutively synthesizing AO in glucose medium (Stasyk *et al.*, 2004) served as a positive control. Interestingly, when methanol was added to glucose medium, the defect in repression in Δ*mig1* and Δ*mig1*Δ*mig2*, but not in Δ*tup1*, became more pronounced (Fig. 4). This suggests that certain transcriptional induction components are induced for AO synthesis in *mig1* cells and may explain the ability of mutants to grow on methanol in the presence of 2-deoxyglucose (Fig. 3A). However, the AO protein level in the double Δ*mig1*Δ*mig2* mutant on carbon source mixture was considerably lower relative to glucose-grown Δ*gcr1* cells or methanol-grown wild-type cells (Fig. 4 and legend).

Similarly, AO synthesis in Δ*mig1*, Δ*mig2*, Δ*mig1*Δ*mig2*, and Δ*tup1* cells was only weakly derepressed in cells exposed to sucrose or ethanol (Fig. 4), suggesting a limited effect of the analysed mutations on the repression pathway(s) in *H. polymorpha*. We excluded peroxisome degradation as a principal factor influencing AO protein level in the analysed mutants grown on multicarbon substrates, as pexophagy is blocked (in *tup1*) or impaired in these strains (see next section).

Hansenula polymorpha Mig1 homologues interfere with pexophagy

Hansenula polymorpha HpTup1 is the only transcriptional repressor shown so far to be essential for macropexophagy (Leao-Helder *et al.*, 2004). We were interested in elucidating whether its possible partners in transcriptional regulation, HpMig1 and HpMig2, also affect this process. In AO activity plate assays, upon a shift of methanol-grown cells to glucose or ethanol, we observed that Δ*mig1* and Δ*mig1*Δ*mig2*, but not Δ*mig2*, cells exhibit enhanced residual AO activities relative to wild-type controls. However, the defect in pexophagy in Δ*mig1* was less pronounced relative to Δ*tup1* cells (not shown). Similarly to the case for Δ*tup1*, no *mig* mutant was significantly affected in general autophagy upon nitrogen starvation, as judged by means of the Phloxine-B plate test (Tsukada & Ohsumi, 1993) (not shown).

Detailed time-course analysis of pexophagy by following AO protein level revealed that the rate of AO degradation differed between the wild-type strain and *mig* mutants when methanol-grown cells were exposed to glucose or ethanol (Fig. 5a). However, Western blots demonstrated that, also during the growth of Δ*mig1*Δ*mig2* cells on methanol, minor AO degradation bands could be observed that were absent in wild-type controls (Fig. 5b), indicating that peroxisomes are degraded and that pexophagy is not completely blocked under these conditions in Δ*mig1*Δ*mig2* cells.

Electron microscopy of methanol-grown Δ*mig1*Δ*mig2* cells exposed to ethanol revealed that peroxisomes indeed undergo degradation and that AO protein can be detected in vacuolar lumen (Fig. 6c). Unexpectedly, whereas in wild-type controls organelles were degraded via macropexophagy (not shown), in Δ*mig1*Δ*mig2* cells the sequestration of organelles to be degraded was rarely observed (Fig. 6b). Instead, the morphological characteristics of micropexophagy, i.e. vacuolar protrusions surrounding peroxisome clusters, were prominent (Fig. 6a).

Discussion

This paper describes the role of *H. polymorpha* transcriptional repressors in catabolite repression and peroxisome

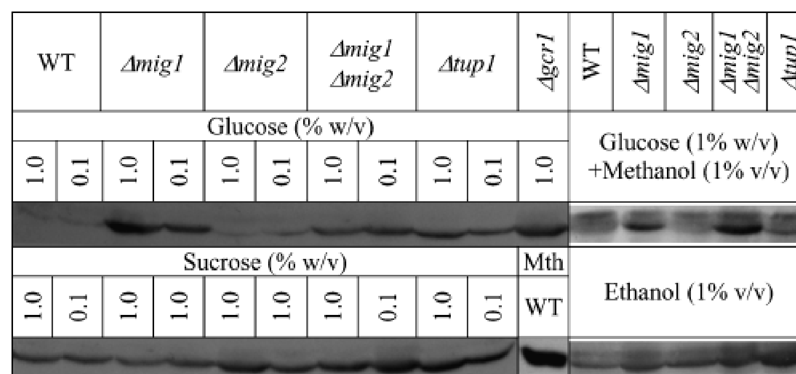

Fig. 4. Analysis of AO synthesis upon incubation of wild-type and mutant strains on various carbon sources. Cells of the indicated *H. polymorpha* strains were pre-grown in the rich YPS medium and shifted to mineral media supplemented with various carbon sources (concentrations are indicated), and then incubated for 6 h. One hundred micrograms of glucose-, sucrose- and ethanol-grown Δ*mig1*, Δ*mig2*, Δ*mig1*Δ*mig2*, Δ*tup1* and 10 μg of glucose-grown Δ*gcr1* TCA extracts of cultures were loaded per lane. Fifty micrograms of methanol-with-glucose-grown cultures were loaded per lane. Five micrograms of methanol-grown wild type culture was loaded per lane to serve as a control [Mth–1% methanol (v/v)]. AO protein was visualized by Western blotting with anti-AO bodies.

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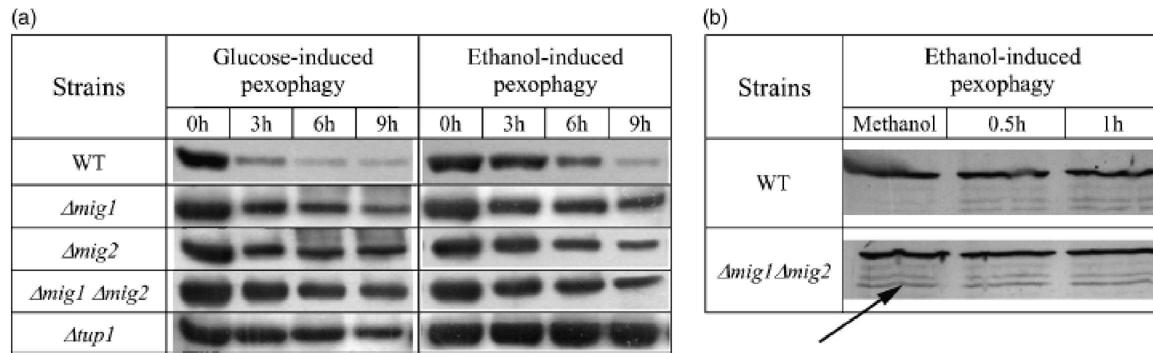

Fig. 5. Kinetic analysis of pexophagy. Cells of the indicated strains were grown on methanol medium and shifted to glucose and ethanol media to induce selective peroxisome degradation. Samples were taken at the indicated time points after the shift. Equal volumes of cultures were loaded per lane and analysed for the presence of AO protein by Western blotting using anti-AO bodies. (a) Kinetics of AO degradation induced by glucose or ethanol. (b) First stages of AO degradation induced by ethanol in wild-type and *Δmig1Δmig2* strains. Notice the appearance of AO degradation products in extracts of *Δmig1Δmig2* cells incubated in methanol before the shift (arrow).

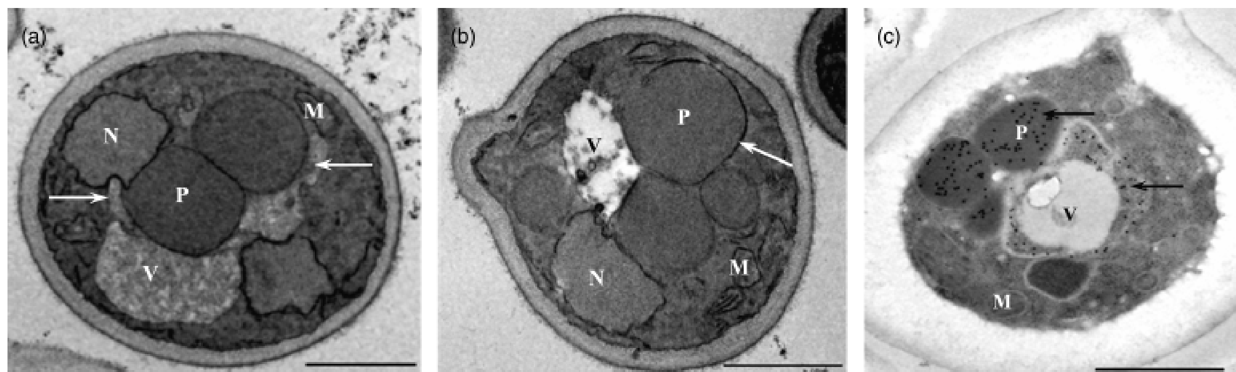

Fig. 6. *Hansenula polymorpha Δmig1Δmig2* cells are disturbed in macropexophagy but not in microautophagy. Methanol-grown *Δmig1Δmig2* cells were exposed to ethanol for 1 h. (a) Peroxisomes are surrounded by vacuolar protrusions, indicative of microautophagy (arrows). (b) A peroxisome is sequestered by additional membrane structures. However, sequestration is not completed (arrow). (c) Immunolabelling reveals that, in methanol-grown *Δmig1Δmig2* cells exposed to ethanol, AO protein is localized both to peroxisomes and to the vacuole, suggesting functional organelle degradation (arrows). M, mitochondrion; V, vacuole; P, peroxisome; N, nucleus. The bar represents 1 μm.

degradation (catabolite inactivation) induced by rich carbon sources. We demonstrated that the repression pathway for peroxisomal AO in this yeast apparently only partially relies on the two Mig1 homologues, irrespective of the carbon source (glucose, sucrose or ethanol). However, addition of the inducer methanol elevates the AO protein level in *Δmig1Δmig2* mutant incubated with glucose (Fig. 4), suggesting that this mutant exhibits a so-called 'inducible' phenotype (Sibirny *et al.*, 1988).

In *S. cerevisiae*, Mig1 is regulated by glucose (Ostling & Ronne, 1998) and facilitates binding of the general repressor complex Tup1-Ssn6 to the promoters of glucose-repressible genes (Gancedo, 1998). However, Tup1 is involved in pleiotropic functions through interaction with specific DNA-binding proteins for each functionally related set of genes (reviewed in Malave & Dent, 2006), while the Mig1-dependent regulation is thought to be more specific, with a limited set of the target genes that include those repressed by glucose (Murad *et al.*, 2001). Therefore, our results are in line with the previous observation that HpTup1 is not required for glucose repression of peroxisomal enzymes (Oliveira *et al.*, 2003), and suggest that, at least for alcohol oxidase, operation of the classical *S. cerevisiae* pathway with Mig1/2-mediated Tup1-Ssn6 binding to the repressible promoter is unlikely. However, the redundant function of these repressors cannot be excluded, and the effect of combining all three mutations in one strain would be interesting to examine. An alternative explanation is the involvement of other still-unknown repressor(s). Therefore, further studies are required to obtain conclusive results on

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whether the effect of *mig1/2* and *tup1* mutations on catabolite repression in response to different carbon sources is direct or indirect. It also has to be investigated whether the analysed mutations interfere with the regulation of other glucose-repressible genes in *H. polymorpha*, for instance maltase, the regulation of which has been suggested to be Mig-dependent (Alamae *et al*., 2003).

The pleiotropic function of Tup1 in yeast was recently extended to regulation of macropexophagy (Leao-Helder *et al*., 2004). The finding that micropexophagy in *H. polymorpha* (Monastyrska *et al*., 2005) and *P. pastoris* (Tuttle & Dunn, 1995) is sensitive to cycloheximide suggests that it is dependent on protein synthesis, and, consequently, on transcriptional regulation.

Our data suggest that the effect of Tup1 deletion on pexophagy is not mimicked by a similar deficiency in HpMig homologues. In *H. polymorpha tup1* mutant, macropexophagy is completely blocked, whereas micropexophagy occurs in the wild-type manner, i.e. only upon nitrogen starvation but not upon a shift of the carbon source (Leao-Helder *et al*., 2004). In the Δ*mig1*Δ*mig2* deletion strain, macropexophagy may also be impaired, whereas micropexophagy seems to be constitutively induced. This resembles the phenotype of *H. polymorpha* Δ*atg25* mutant, which also displayed constitutive microautophagy in conjunction with a block in macropexophagy (Monastyrska *et al*., 2005). This apparent similarity of the phenotypes of *H. polymorpha* Δ*mig1*Δ*mig2* and Δ*atg25* mutants suggests that certain macropexophagy-specific components may be down-regulated in the absence of Mig repressors, and, hence, Mig repressors may be positive regulators of macropexophagy. Further research to elucidate the details of these effects is required, including the use of microarray approaches.

With regard to the control of micropexophagy, Mig1 homologues and Tup1 seem to have distinct roles depending on environmental conditions. Mig1 proteins may be negative regulators of micropexophagy, whereas Tup1 acts as a positive regulator, but only upon a shift in the carbon source and not upon nitrogen depletion conditions. A tentative model explaining such regulations is presented in Fig. 7.

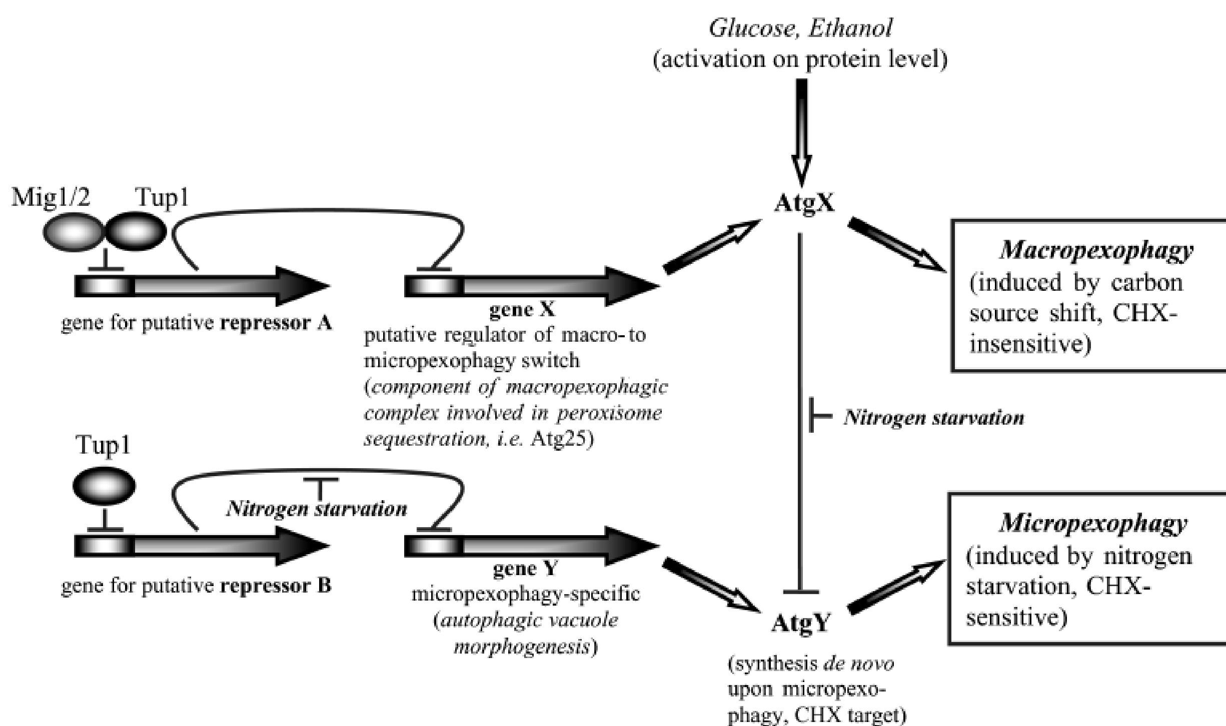

Fig. 7. Tentative model of the involvement of HpMig- and HpTup1-mediated transcriptional repression in the regulation of pexophagy. We suggest that certain components of macropexophagy-specific machinery (such as HpAtg25) may be negative regulators of micropexophagy. Mig1/2 and Tup1 are indirect positive regulators of such hypothetical proteins (AtgX), which are activated posttranslationally by the effectors of macropexophagy, glucose and ethanol, and inhibit the hypothetical micropexophagic component AtgY. AtgX level in the absence of either Mig1/2 or Tup1 is decreased. Nitrogen starvation confers positive regulation of AtgY by inducing its expression and repressing macropexophagy at the protein level. In the absence of Tup1, but not Mig1/2, micropexophagy occurs only upon nitrogen starvation. We suggest that AtgY may be involved in the development of microautophagic vacuoles. CHX, cycloheximide.

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