The Nutrient Stress-induced Small GTPase Rab5 Contributes to the Activation of Vesicle Trafficking and Vacuolar Activity*

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Background: How the function of Rab5 isoforms is regulated remains unclear.

Results: The third Rab5 isoform, Ypt53, is up-regulated significantly under nutrient stress.

Conclusion: The up-regulated Ypt53 and the constitutive Vps21 are crucial for vesicle transport and vacuolar hydrolase activity, and they prevent ROS accumulation and mitochondrial dysfunction.

Significance: The function of three Rab5 isoforms may be regulated differently to adapt to environmental changes.

Rab family small GTPases regulate membrane trafficking by spatiotemporal recruitment of various effectors. However, it remains largely unclear how the expression and functions of Rab proteins are regulated in response to extracellular or intracellular stimuli. Here we show that Ypt53, one isoform of Rab5 in Saccharomyces cerevisiae, is up-regulated significantly under nutrient stress. Under non-stress conditions, Vps21, a constitutively expressed Rab5 isoform, is crucial to Golgi-vacuole trafficking and to vacuolar hydrolase activity. However, when cells are exposed to nutrient stress for an extended period of time, the up-regulated Ypt53 and the constitutive Vps21 function redundantly to maintain these activities, which, in turn, prevent the accumulation of reactive oxygen species and maintain mitochondrial respiration. Together, our results clarify the relative roles of these constitutive and nutrient stress-inducible Rab5 proteins that ensure adaptable vesicle trafficking and vacuolar hydrolase activity, thereby allowing cells to adapt to environmental changes.

Eukaryotic cells are compartmentalized into multiple distinct membrane-bound organelles, and elaborate systems are required to transfer proteins and lipids between these compartments. Members of the Rab family of small GTPases are major regulators of macromolecular trafficking in the secretory and endocytic pathways (1–4). Rab GTPases function as molecular switches that alternate between two conformational states: the GDP-bound “off” form and the GTP-bound “on” form. Conversion of the GDP-bound Rab into the GTP-bound form is mediated through the exchange of GDP for GTP, which is catalyzed by a guanine nucleotide exchange factor. The GTP-bound “active” Rabs bind effector proteins and complexes that execute diverse functions in membrane trafficking. The active Rabs are converted back to the GDP-bound “inactive” form by hydrolysis of GTP, which is stimulated by a GTPase-activating protein. Rabs are anchored to membranes through C-terminal prenyl anchors. In their inactive GDP-bound state, Rabs can be extracted from membranes and relocated by the cytosolic chaperone GDP dissociation inhibitor, which binds the prenyl groups of Rabs and delivers them to their target membrane.

In both yeast and mammals, there are three Rab5 isoforms (Vps21, Ypt52, and Ypt53 in yeast and Rab5a, Rab5b, and Rab5c in mammals) that reside on endosomes. Rab5 regulates the transport of proteins from early to late endosomes and then to terminal lysosomes (vacuoles) by controlling vesicle budding, uncoating, motility, tethering, and fusion (5-10). More recently, Rab5 has been shown to be necessary for the biogenesis of the endolysosomal system in vivo (11). The multitasking ability of Rab GTPases is underscored by their spatiotemporal coordination of effector proteins, such as sorting adaptors, tethering factors, kinases, phosphatases, and various regulators of membrane trafficking. For example, the Vps21-mediated tethering activity may be promoted by the class C core vacuole/endosome tethering complex and also regulated by upstream activators, inhibitors, and effectors (9, 12–15). However, in contrast to the accumulated knowledge on the catalytic activity of Vps21, much less is known about the mechanisms by which the functions of other Rab5 isoforms are regulated (16, 17). In particular, our understanding of the molecular mechanisms controlling the expression of Rab proteins is still limited, although their altered expression has now been analyzed in cells of the immune system (18).

In a previous study, we demonstrated that the activity of Ypt52 is regulated negatively by Roy1, a non-Skp1-Cull1-F-box-type F-box protein. In this study, we demonstrate that another isoform of Rab5 in Saccharomyces cerevisiae, Ypt53, is up-regulated significantly under nutrient stress conditions. We found that constitutively expressed Vps21 plays a major role in vesicle trafficking under normal conditions and in the activation of vacuolar hydrolase activity. However, when cells are exposed to nutrient stress for an extended period of time, Ypt53 is up-
regulated significantly and functions redundantly with Vps21 to facilitate intracellular trafficking. Moreover, Ypt53 and Vps21 are both critical to prevent the accumulation of reactive oxygen species ROS and to maintain mitochondrial respiration. Therefore, we propose that Ypt53 functions as a backup factor that confers robustness against nutrient stress by expanding Rab5 signaling.

**EXPERIMENTAL PROCEDURES**

*Strains and Culture Conditions—* Yeast strains were constructed by PCR-based homologous recombination. The strains used in this study were as follows: W303-1a (MATa, can1-100, leu2-3, -112, his3-11, -15, trp1-1, ura3-1, ade2-1), TK2262 (W303-1a, pdr5Δ:HPH, vps21Δ:TRP1) (19), KNY344 (W303-1a, pdr5Δ:HPH, ypt53Δ:LEU2), KNY345 (W303-1a, pdr5Δ:HPH, vps21Δ:TRP1, ypt53Δ:LEU2), KNY387 (W303-1a, pdr5Δ:HPH, vps21Δ:TRP1, ypt52Δ:HAS3, ypt53Δ:LEU2), KNY140 (W303-1a, pdr5Δ:HPH, pep4Δ:LEU2) (20), KNY164 (W303-1a, pdr5Δ:HPH, pep4Δ:LEU2, atg1Δ:CgHIS3), BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0), and KNY15-8D (BY4741, gis1Δ1:KanR).

Cells were grown in YP-rich medium (1% yeast extract, 1% peptone, and 100 mg/liter of adenine hydrochloride) containing 2% glucose, 2% glycerol, or 2% ethanol. When the petite frequency was calculated, adenine hydrochloride was removed from the YPglucose agar plate. Cells harboring plasmids were cultured in synthetic complete medium (SD) (0.67% yeast nitrogen base without amino acids, 2% glucose, and 100 mg/l of adenine hydrochloride) supplemented with appropriate amino acids (dropout mix) (21). Nitrogen was starved either in SD-N medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, and 50 mM MES-KOH (pH 6.2)) (22) or S-N medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, and 50 mM MES-KOH (pH 6.2)). Autophagic bodies were observed after cells were shifted to S-N medium supplemented with 1 mM PMSF for 3 h. Nomarski image, GFP, and FM4-64 images were captured by Axio Observer.Z1 (Carl Zeiss), and images were analyzed with AxiosVision 4.6.

*Plasmids—* The plasmid encoding Candida glabrata HIS3 gene (pUC19-CgHIS3) was provided by the National Bio-Resource Project of MEXT, Japan. The plasmid encoding YPT53 under the control of the VPS21 promoter was constructed as follows. The VPS21 promoter (1000 bp) and the open reading frame of the YPT53 gene were amplified by PCR and cloned into pRS316. The plasmid encoding GFP-Atg8 was constructed as follows. The promoter region of ATG8 (1000 bp), the gene for enhanced GFP (derived from pTYE481, a gift from T. Yoshihisa, University of Hyogo, Hyogo, Japan), and the open reading frame and the terminator (1000 bp) of ATG8 were inserted into pRS313 to give P<sub>ATG8</sub>-GFP-ATG8-T<sub>ATG8</sub>. A pRS316-based plasmid encoding P<sub>ATG8</sub>-GFP-ATG8-T<sub>ATG8</sub> was also used (a gift from H. Nakatogawa and Y. Ohsumi, Tokyo Institute of Technology, Kanagawa, Japan). All primer sequences are available upon request.

*RT-PCR—* Isolation of total RNA and cDNA synthesis were performed using TriPure isolation reagents (Roche) and ReverTra Ace (ToyoBo), respectively. All primer sequences are available upon request.

*Antibodies and Immunoblot Analysis—* Polyclonal antibodies to Ypt53 were generated in a rabbit by standard procedures with recombinant Ypt53 as an antigen. The anti-Vps21 and Ypt52 antibodies have been described previously (19). The mouse mAb to Pgk1 was purchased from Invitrogen. The rabbit polyclonal antibody to CPY was a gift from T. Endo (Nagoya University, Nagoya, Japan). The rabbit polyclonal antibody to Ape1p was a gift from H. Nakatogawa and Y. Ohsumi (Tokyo Institute of Technology, Kanagawa, Japan).

The concentrations of Ypt53 and Vps21 in cells were estimated as follows. His<sub>6</sub>-tagged Ypt53 and Vps21 were expressed from pET30a (Novagen) in JM109 (DE3) and purified by nickel-nitrilotriacetic acid-agarose (Wako). These purified proteins were used as a standard to calculate the amount of Ypt53 and Vps21 in whole cell lysate by Western blotting with anti-Ypt53 and anti-Vps21 antibodies. The amount of each protein in 1 A<sub>600</sub> equivalent of cells was calculated.

*Detection of Carbonylated Proteins—* Carbonylated proteins on the PVDF membrane were reacted with 2,4-dinitrophenyl hydrazine and detected by Western blotting with anti-2,4-dinitrophenyl hydrazine rabbit polyclonal antibodies (Shima Laboratories Co., Ltd., catalog no. ROIK03) (23). Briefly, the total yeast cell lysate was separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was soaked in 100% methanol (1 min), 20% methanol and 80% TBS (5 min), 2 N HCl (5 min), 2,4-dinitrophenyl hydrazine solution (5 min), 2 N HCl (5 min, three times), 100% methanol (5 min, seven times), and 100% TBS (5 min). This membrane was blocked with 5% skim milk in TBST (TBS supplemented with 0.1% Tween 20) and then incubated with anti-2,4-dinitrophenyl hydrazine antibodies. Carbonylated proteins were detected by standard Western blotting procedures.

**RESULTS**

*Expression of Ypt53 Is Induced under Nutrient Stress Conditions—* Previous reports have demonstrated that ypt53Δ cells do not show any clear phenotypes under normal growth conditions (5, 15). Other reports implied that the expression of Ypt53 is lower than that of Vps21 and Ypt52 (24). During our analysis of endogenous Rab5 proteins using specific antibodies, we found a significant increase in Ypt53 over the time when cells were grown to post-log phase in glucose-containing medium (Fig. 1, A and B, lanes 2–5), although it was almost undetectable during log phase (lane 1). Yeast cells preferentially ferment glucose to produce ATP but can also metabolize a non-fermentable carbon source, such as glycerol or ethanol, for ATP production. When cells exhaust glucose in medium, they start to use ethanol that accumulates from the prior fermentation of glucose. This conversion is termed the “diauxic shift.” The up-regulation of Ypt53 in glucose-grown cells is most likely triggered by the diauxic shift because we observed a dramatic increase in the expression of Ypt53, but not of Vps21 and Ypt52, when the carbon source was shifted from glucose to glycerol or ethanol (Fig. 1C). The induction of Ypt53 in glycerol-grown...
cells appeared to occur at the transcriptional level (Fig. 1D). In addition to the induction of Ypt53 at the diauxic shift, we also noted that the level of Ypt53 continued to increase over time, even after cells entered the stationary phase (Fig. 1B, >48 h). At the stationary phase, cells exhaust available carbon sources and are exposed to nutrient stress (25). Indeed, the induction of Ypt53 at the stationary phase was dependent on Gis1 (Fig. 1E), a transcription factor that is known to regulate genes during nutrient limitation (26). Together, these results suggest that the expression of Ypt53 is induced under nutrient stress conditions. As far as we know, this is the first instance of Rab5 GTPase expression being regulated by nutrient status.

Role of Ypt53 and Vps21 in Vesicle Trafficking at Post-log Phase—To investigate the role of the induced Ypt53 in vesicle trafficking, we monitored the maturation of carboxypeptidase Y (CPY), a soluble vacuolar marker protein (27). In the endoplasmic reticulum (ER), a prepro form of CPY undergoes proteolytic cleavage and addition of N-linked oligosaccharide chains to generate a 67-kDa ER form, “p1CPY.” In the Golgi complex, p1CPY is converted to a 69-kDa form, “p2CPY,” by addition of mannose to the N-linked glycans. After reaching the vacuole, p2CPY becomes a 61-kDa mature form, “mCPY.” As shown in Fig. 2A, when wild-type cells were grown in glucose-containing medium to early log phase (A600 ~0.5), CPY was detected as the mature form (lane 1, α-CPY blot). Similarly, in ypt53Δ cells, CPY was transported normally to the vacuole (Fig. 2A, lane 2). In contrast, in vps21Δ cells, the total amount of CPY decreased, and the p1 and p2 forms accumulated (Fig. 2A, lane 3). This is consistent with previous reports that a vast majority of CPY is missorted into the extracellular space upon deletion of VPS21. A similar result was obtained for vps21Δypt53Δ cells (Fig. 2A, lane 4). When cells were grown to post-log phase (>60 h of culture), CPY was still detected as a mature form in wild-type and ypt53Δ cells (Fig. 2A, lanes 5 and 6). Surprisingly, a significant portion of CPY was also delivered to the vacuole, even in vps21Δ cells (Fig. 2A, lane 7). Additional deletion of YPT53 in vps21Δ cells prevented the transport of CPY to the vacuole (Fig. 2A, lane 8). Similar results were obtained when cells were grown in glycerol-containing medium (Fig. 2A, lanes 9–16). These results suggest that the induced Ypt53 during post-log phase can contribute to Golgi-vacuole protein transport under nutrient stress conditions.

We next investigated the relative concentrations of Ypt53 and Vps21 during post-log phase. We found that the amount of Ypt53 was 10–15-fold less than that of Vps21. The concentration of Ypt53 was estimated to be ~5 ng/1.0 A600 cells, and that of Vps21 was ~50 ng/1.0 A600 cells. This difference is in line with the fact that the loss of Ypt53 did not cause any defect in CPY transport during post-log phase (Fig. 2A, lanes 6 and 14), whereas the loss of Vps21 caused a slight defect in this event (lanes 7 and 15). Together, although the contribution of Vps21 is still larger than that of Ypt53, these two Rab5 GTPases may function redundantly and support the vacuolar transport of CPY during post-log phase.

The idea for the functional redundancy between Vps21 and Ypt53 can be supported by the fact that they share the highest
sequence similarity in three Rab5 isoforms in yeast (5). Vps21 and Ypt53 share ~57% identity in amino acid sequence, whereas Vps21 and Ypt52 share ~48% identity, and Ypt52 and Ypt53 share ~53% identity. Moreover, we observed a synthetic growth defect of cells lacking both Ypt53 and Vps21 during post-log phase in glucose- and glycerol-containing medium (Fig. 2B). Finally, when Ypt53 was overexpressed from a plasmid under the control of the VPS21 promoter, it successfully rescued the transport defect of CPY in the vps21Δypt52Δypt53Δ triple mutant cells (Fig. 2C). These results suggest that Ypt53 has an overlapping function with Vps21.

Ypt53 Functions Redundantly with Vps21 to Help Strengthen Vacuolar Hydrolase Activity under Nutrient-limited Conditions—Autophagy is a nonspecific degradation process that is highly conserved among eukaryotes (28–30). Under nutrient-limited conditions, cytosolic double-membrane vesicles emerge and sequester cytosolic proteins and organelles as cargo, and then deliver this cargo to the lysosomes/vacuoles for degradation (28–30). Because the expression of Ypt53 turned out to be related to the nutrient status of the cell, we wondered whether its expression might also be induced under nitrogen starvation conditions, where vacuolar hydrolase activity is necessary for autophagy. Strikingly, as shown in Fig. 3A, Ypt53 was strongly up-regulated when cells were shifted from rich medium to nitrogen starvation medium. The levels of Vps21 and Ypt52 were unchanged under the same conditions.

Because depletion of Vps21 and Ypt53 resulted in a major defect in the delivery of CPY to the vacuole, we reasoned that the up-regulated Ypt53 might contribute to strengthen vacuolar hydrolase activity under nutrient-limited conditions. To investigate this possibility, we monitored the processing of GFP-Atg8. Upon delivery of GFP-Atg8 to the vacuole via the autophagy pathway, Atg8 was degraded rapidly by proteinases in the vacuole, whereas the released GFP remained relatively stable (31, 32). In wild-type and ypt53Δ cells, the amounts of free GFP increased over time after cells were shifted to nitrogen starvation medium (Fig. 3B, lanes 1–8). In contrast, no free GFP was detected in vps21Δ and vps21Δypt53Δ cells, suggesting a crucial role of Vps21 in the activation of vacuolar hydrolase.

In parallel with GFP-Atg8, we monitored the processing of aminopeptidase 1 (prApe1) (31, 33). The precursor form of Ape1 that is synthesized in the cytosol is delivered to the vacuole via the cytosol-to-vacuole targeting pathway under nutrient-rich conditions and through autophagy under nitrogen starvation conditions (34). After entering the vacuole, the N-terminal propeptide of prApe1 is cleaved to generate the mature form (mApe1). The maturation of prApe1 appeared to be normal in ypt53Δ cells compared with wild-type cells under rich conditions (Fig. 3B, lanes 1 and 5) and under nitrogen starvation conditions (Fig. 3B, lanes 2–4 and 6–8). However, in vps21Δ and vps21Δypt53Δ cells, mature Ape1 was almost undetectable, even under rich conditions (Fig. 3B, lanes 9 and 13; see also Fig. 3C, lanes 5 and 7). A defect in Ape1 maturation under nutrient-rich conditions in these mutants may be due to a block in the cytosol-to-vacuole targeting pathway and/or an inefficient cleavage of the Ape1 propeptide in the vacuole by luminal proteases that were transported into the vacuole inefficiently because of the depletion of Rab5 proteins (Fig. 2A). When these cells were shifted to nitrogen-starved medium, only a slight amount of mature Ape1 was observed in vps21Δ cells, and its degree was indistinguishable from that in vps21Δypt53Δ cells (Fig. 3B, lanes 10–12 and 14–16). These results demonstrate that, in contrast to our initial expectation, the contribution of Ypt53 to facilitating the vacuolar hydrolase activity is only limited, whereas Vps21 plays a major role in its activity, at least during the period shortly after cells were shifted to nitrogen starvation conditions.

However, when cells were incubated under nitrogen starvation conditions for an extended period of time (~24 h), we noticed that Ypt53 continued to increase over time (Fig. 3C, lanes 1–2). Importantly, after prolonged incubation, GFP-Atg8 and prApe1 were processed eventually, even in vps21Δ cells (Fig. 3C, lane 6), whereas the processing of these marker proteins was reduced strongly in vps21Δypt53Δ cells (Fig. 3C, lane 8). The induction of Ypt53 under nitrogen-starvation condition could also partially rescue the CPY transport defect in vps21Δ cells (Fig. 3D, compare lanes 6 and 8). The partial rescue of the vps21Δ phenotype by Ypt53 can be explained by the relative concentration of Vps21 and Ypt53. The amount of Ypt53 and Vps21 proteins under nitrogen starvation conditions was estimated to be ~9 ng/1.0 A600 cells and ~45 ng/1.0 A600 cells, respectively.
Although these results suggest that the transport defect of GFP-Atg8 and prApe1 was caused by the incomplete transport of vacuolar proteins, it is still possible that the autophagy pathway is somehow impaired in vps21/H9004 ypt53/H9004 cells. To test this possibility, we first analyzed the autophagic bodies in vacuoles. As shown in Fig. 3E, autophagic body-like granular structures were observed in the vacuole of vps21Δypt53Δ cells after incubation under starvation conditions (Fig. 3E). Interestingly, these structures appeared to be less motile than autophagic bodies in pep4Δ cells (data not shown). To further analyze the autophagy pathway, we observed the localization of GFP-Atg8. As shown in Fig. 3F, when wild-type cells were shifted to nitrogen starvation conditions, GFP-Atg8 was transported into the vacuole, and the subsequently released free GFP was diffused throughout the vacuole lumen. In hydrolase-deficient cells such as pep4/H9004, it has been well established that autophagic bodies are not broken down and that they cluster together. Indeed, GFP-Atg8 accumulated in the vacuole lumen (32). We then analyzed GFP-Atg8 in vps21/H9004 ypt53/H9004 mutant cells. Interestingly, we observed multiple granular structures of GFP-Atg8. These structures seemed to be distributed throughout the vacuole and looked different from the staining pattern in pep4Δ.
cells, where GFP-Atg8 accumulates and forms relatively small structures in the vacuole. It should be noted that a vacuolar marker, FM4-64 dye (35), reached the vacuole less efficiently in vps21Δypt53Δ cells, probably because this strain shows a strong defect in endocytosis.

One possible scenario that could explain these observations is that the formation and transport of the autophagosome may be unaffected in vps21Δypt53Δ cells. However, subsequent steps (i.e., the release of the autophagic body from the vacuolar membrane into the lumen) might somehow be impaired. This could be one reason for the lower motility of the structure as well as the processing defect of GFP-Atg8. At the same time, it is still possible that the processing defect of GFP-Atg8 was caused by the defect in the vacuolar hydrolase activity. We believe that these two possibilities can occur simultaneously and not in a mutually exclusive manner. Regardless, these observations should form a basis for further investigation of a potentially direct role of Rab5 in autophagy in yeast.

**Nutrient Stress-induced Ypt53 and the Constitutively Expressed Vps21 Function Together to Prevent the Accumulation of ROS and to Maintain Mitochondrial Respiratory Activity**—Previous studies have suggested that defects in autophagy cause the accumulation of ROS (22, 36–38). The reason for ROS accumulation may well be explained by the imbalance of mitochondrial respiratory enzymes, inefficient expression of ROS scavenger proteins, and/or defects in the autophagic degradation of mitochondria (mitophagy) (22, 38). Therefore, we asked whether depletion of Vps21 and Ypt53 might cause the enhanced carbonylation, a non-enzymatic protein modification catalyzed by ROS (39). As shown in Fig. 4, when cells were grown to post-log phase (>70 h), carbonylated proteins accumulated slightly in vps21Δ cells (lane 3), and a significant amount of them accumulated in vps21Δypt53Δ cells (lane 4) compared with wild-type and ypt53Δ cells (lanes 1 and 2). This result supports our idea that Vps21 and Ypt53 have an overlapping function in decreasing ROS under nutrient-limited conditions.

ROS accumulation and/or defects in vacuolar activities are known to lead to the loss of mitochondrial DNA and result in a respiratory-deficient phenotype (40, 41). To test whether deletion of VPS21 and YPT53 might cause a respiratory-deficient (petite) phenotype under nutrient-limited conditions, we took advantage of the ade2-1 mutation of W303 strains. Yeast cells with mutations in the ADE2 gene (W303 strains) accumulate a red pigment because of the disruption of the adenine biosynthetic pathway on YPGlycerol plates. However, yeast cells without functioning mitochondria like "petite" do not reach this step in the adenine synthesis pathway and, thus, form white colonies (42, 43). When cells were cultured under nitrogen starvation conditions for 2 days, only 4~8% of wild-type, ypt53Δ, and vps21Δ cells formed white colonies (Fig. 5, A and B). In contrast, >30% of vps21Δypt53Δ cells formed white colonies. White colonies were streaked on YPGlycerol plates and were confirmed to be respiratory-deficient (Fig. 5C). On the basis of these genetic and biochemical studies, we propose that the constitutively expressed Vps21 and the nutrient stress-induced Ypt53 function together to maintain vacuolar hydrolase activities, which prevent the accumulation of ROS and maintain mitochondrial respiration, demonstrating the physiological importance of the up-regulation of Ypt53 (Fig. 6).

**DISCUSSION**

In this study, we discovered an additional layer of regulation of Rab5 signaling in yeast in which an isoform of Rab5, Ypt53, is up-regulated under nutrient stress conditions. To our knowledge, this is the first example of Rab5 GTPase expression being controlled by nutrient status. Under nutrient-rich conditions, the constitutively expressed Vps21 plays a critical role in vesicle transport and in facilitating the vacuolar hydrolase activities. However, when cells are shifted to nutrient-limited conditions, the constitutive Vps21 and the induced Ypt53 function redundantly and facilitate these activities. Moreover, the function of both Vps21 and Ypt53 is essential for preventing the accumulation of ROS and for maintaining mitochondrial respiratory activity under nutrient-limited conditions (Fig. 6). Therefore, we propose that the up-regulated Ypt53 functions in a “stage-specific” manner that is dependent on nutrient status and helps strengthen net Rab5 activity, thereby allowing cells to adapt to environmental changes.

Cells have evolved various mechanisms to adapt to different nutrient conditions. Autophagy is one such mechanism that sequesters cytosolic cargo for degradation in the vacuole. Autophagy is not only a housekeeping process, but it is also activated under nutrient-limited conditions to provide supplementary reserves for the starving cells (44). However, during nutrient starvation, the influx of cargo (proteins and lipids) into the vacuole by autophagy exerts increasing pressure on the degradation systems in the vacuole. To overcome this pressure, the hydrolytic capacity of the vacuole should also be increased under nutrient-limited conditions. The up-regulated Ypt53 may well contribute to the increase in vacuolar hydrolase activity, most likely by facilitating the transport of vacuolar enzymes. In addition, we have shown that the up-regulation of Ypt53 is
mediated, at least in part, by the transcription factor Gis1 (Fig. 1E). Gis1 is known to be negatively regulated by Tor1 (target of rapamycin) (45). Therefore, we propose that, under nutrient-limited conditions, Tor1 is inactivated and that the activated Gis1 may up-regulate Ypt53. In this regard, it is interesting to note that Rab5 proteins both in yeast and mammals regulate the activity and localization of TORC1 (16). One attractive hypothesis is that the up-regulated Rab5 signaling under nutrient limited conditions functions as a positive feedback mechanism that modulates TORC1 activity and its downstream signaling cascade.

Both Vps21 and Ypt53 have been shown to bind to Vps8 (12, 13), which is one of the effector proteins that associate with class C core vacuole/endosome tethering, suggesting that Vps21 and Ypt53 may share downstream effector proteins. Consistent with this notion, the up-regulated Ypt53 could partially rescue the vps21Δ phenotype (i.e. the intracellular transport and the activation of vacuolar hydrolase activities) under nutrient stress conditions. The functional redundancy between Vps21 and Ypt53 is also underscored by the fact that overexpressed Ypt53 from the VPS21 promoter rescued the defect of CPY trafficking in vps21Δypt52Δypt53Δ triple mutant cells (Fig. 2B). In the case of another Rab5 isoform, Ypt52, the

FIGURE 5. Ypt53 and Vps21p are required to maintain the respiratory activity of mitochondria during prolonged incubation of cells in nitrogen starvation medium. A, cells were grown in synthetic complete medium, transferred to SD-N + 50 mM MES-KOH medium (pH 6.2), and incubated further for 2 days. Subsequently, cells were spread on a YPglucose agar plate (minus adenine), and the plates were incubated for 4–5 days. Cells that contain ade2 mutations accumulate a red pigment on the YPglucose plate. However, when cells lack respiratory activity, they form white colonies. B, the percentage of white colonies was calculated. The bar graph shows mean ± S.D. of three independent experiments (error bars). C, cells from red and white colonies were streaked on YPglucose and YPGlycerol plates to test the respiratory defect. Representative plates are shown. Red colonies were able to grow on both YPglucose and YPGlycerol plates, whereas white colonies were able to grow on the YPglucose plate but not on the YPGlycerol plate, indicating that cells in white colonies are respiratory-deficient.

FIGURE 6. A possible model for the physiological role of the nutrient stress-induced Ypt53 and constitutive Vps21 in vacuolar function. See text for details.
Rab5 Isoform Regulation under Nutrient Stress Conditions

GTTPase activity is negatively regulated by Roy1, a non-Skp1-Cul1-F-box-type F-box protein, under normal conditions (19). It is currently unknown how this negative regulation is canceled. However, the deletion of Roy1 could also partially rescue the vps2Δ phenotypes (19), implying a potential role of Ypt52 in class C core vacuole/endosome tethering. In fact, GTP-locked Ypt52 has also been shown to bind Vps8 (13). It should be noted that the levels of Ypt52 and Roy1 were unaltered under nutrient-limited conditions (data not shown). Therefore, the activation of Ypt52 might occur under conditions other than nutrient stress.

Although it is quite likely that the up-regulated Ypt53 contributes to the activation of vacuolar activity, our results also raised the possibility that Rab5 might function in the release of the autophagic body from the vacuolar membrane into the lumen. It has been proposed that, in mammals, autophagy can be activated downstream of Rab5 signaling through the beclin1-Vps34-containing complex (46, 47). Further analysis is required to clarify a potential direct role of Rab5 in both yeast and mammals, but we consider that the Rab5-dependent activation of vacuolar activity and Rab5-mediated completion of autophagy can occur simultaneously and not in a mutually exclusive manner. In any event, our observations should form a basis for further investigation of a role of Rab5 in vacuolar activities.

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