

ORIGINAL RESEARCH

Components of Golgi-to-vacuole trafficking are required for nitrogen- and TORC1-responsive regulation of the yeast GATA factors

Mohammad Fayyadkazan^{1,2}, Jennifer J. Tate³, Fabienne Vierendeels¹, Terrance G. Cooper³, Evelyne Dubois¹ & Isabelle Georis¹

¹Institut de Recherches Microbiologiques J.-M. Wiame, Laboratoire de Microbiologie, Université Libre de Bruxelles, 1070 Brussels, Belgium

²Laboratoire de Biologie du Transport Membranaire, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 6041 Gosselies, Belgium

³Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee 38163

Keywords

GATA factor, nitrogen availability, rapamycin, vesicular trafficking, yeast.

Correspondence

Isabelle Georis, IRMW, Yeast Physiology, Av. E. Gryzon, 1, 1070 Brussels, Belgium.
Tel: +322-526-7277; Fax: +322-526-7273;
E-mail: igeoris@ulb.ac.be

Funding Information

This work was supported by the Commission Communautaire Française (COCOF; E. D., F. V., and I. G.) and the Fonds de la Recherche Fondamentale Collective (FRFC 2.4547.11; E. D. and I. G.) and National Institute of General Medical Sciences grant GM-35642 (J. J. T. and T. G. C.). M. F. K. is an FNRS Research Fellow (Fonds de la Recherche Scientifique).

Received: 26 November 2013; Revised: 20 January 2014; Accepted: 27 January 2014

MicrobiologyOpen 2014; 3(3): 271–287

doi: 10.1002/mbo3.168

Introduction

Nitrogen is a naturally occurring element that is essential for the growth of all living cells. Many microorganisms have the ability to sense and utilize a broad range of nitrogen sources. When *Saccharomyces cerevisiae* cells are exposed to preferred nitrogen sources (ammonia and glutamine), the expression of genes encoding proteins required for the uptake and utilization of nonpreferred sources (proline, urea, and allantoin) are downregulated.

Abstract

Nitrogen catabolite repression (NCR) is the regulatory pathway through which *Saccharomyces cerevisiae* responds to the available nitrogen status and selectively utilizes rich nitrogen sources in preference to poor ones. Expression of NCR-sensitive genes is mediated by two transcription activators, Gln3 and Gat1, in response to provision of a poorly used nitrogen source or following treatment with the TORC1 inhibitor, rapamycin. During nitrogen excess, the transcription activators are sequestered in the cytoplasm in a Ure2-dependent fashion. Here, we show that Vps components are required for Gln3 localization and function in response to rapamycin treatment when cells are grown in defined yeast nitrogen base but not in complex yeast peptone dextrose medium. On the other hand, Gat1 function was altered in *vps* mutants in all conditions tested. A significant fraction of Gat1, like Gln3, is associated with light intracellular membranes. Further, our results are consistent with the possibility that Ure2 might function downstream of the Vps components during the control of GATA factor-mediated gene expression. These observations demonstrate distinct media-dependent requirements of vesicular trafficking components for wild-type responses of GATA factor localization and function. As a result, the current model describing participation of Vps system components in events associated with translocation of Gln3 into the nucleus following rapamycin treatment or growth in nitrogen-poor medium requires modification.

Conversely, when provided with poorly utilized nitrogen sources, expression of these genes is derepressed. The regulatory pathway responsible for this behavior is known as *nitrogen catabolite repression* (NCR) (Cooper 1982; Wiame et al. 1985). Expression of NCR-sensitive genes is mediated by two DNA-binding GATA transcription activators, Gln3 and Gat1/Nil1 (Mitchell and Magasanik 1984; Coffman et al. 1995, 1996; Stanbrough et al. 1995), and is inhibited by the preprion protein Ure2, which acts as a negative regulator of Gln3 and Gat1 (Drillien and

Lacroute 1972; Grenson *et al.* 1974; Courchesne and Magasanik 1988; Blinder *et al.* 1996; Coffman *et al.* 1996). In the presence of preferred nitrogen sources, Gln3 and Gat1 are sequestered in the cytoplasm in a Ure2-dependent manner, whereas upon growth in a poor nitrogen supply or upon transferring to nonpreferred nitrogen conditions, the GATA activators relocate to the nucleus and mediate the transcription of NCR-sensitive genes (Cox *et al.* 2000; Cunningham *et al.* 2000; Cooper 2002).

The nutrient-responsive TOR complex 1 (TORC1) was also found to participate in coordinating the regulation of GATA factor-mediated expression. Indeed, addition of the immunosuppressant drug rapamycin, inhibiting TORC1, to cells growing in the presence of a good nitrogen source transiently mimics the effects observed with a poor one, that is, nuclear localization and activation of NCR gene expression by Gln3 and Gat1 (Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999). However, a growing literature demonstrates that multiple distinct regulatory mechanisms are involved in the control of the GATA activators and hence results observed in response to rapamycin treatment cannot be extrapolated to explain responses to nitrogen limitation (Cox *et al.* 2004; Tate *et al.* 2005, 2006, 2010; Puria *et al.* 2008; Georis *et al.* 2011a; Feller *et al.* 2013; Rai *et al.* 2013b; Tate and Cooper 2013). Moreover, although Gln3 and Gat1 are both required for the transcription of most NCR-sensitive genes, the two transcription factors are not always similarly regulated: (1) Gln3 and Gat1 localizations are not similarly sensitive to nitrogen limitation, rapamycin and methionine sulfoximine (Mxs, an inhibitor of glutamine synthetase) treatment (Tate *et al.* 2010; Georis *et al.* 2011a). (2) In contrast to Gln3, Gat1 nuclear localization is largely Ure2-independent (Georis *et al.* 2008). (3) Nuclear localization of Gln3 and Gat1 exhibits distinct requirements for the TORC1-regulated phosphatases (Tate *et al.* 2010; Georis *et al.* 2011b).

Early investigations of Gln3 regulation established that under steady state, nitrogen-rich conditions, cytoplasmic Gln3 was situated within or tightly associated with a cytoplasmic membrane system (Cox *et al.* 2002). This membrane association was additionally visualized as Gln3 translocated into and out of the nucleus (Cox *et al.* 2004). Puria *et al.* (2008) subsequently confirmed the Gln3 membrane association by demonstrating colocalization of Gln3-Myc¹³ with the Golgi-to-endosome trafficking component Vps10-HA. Endogenous membranes of the protein secretory pathway have also emerged as important platforms for Tor signaling. First, all components of the TORC1 complex (Tor1, Tor2, Kog1, Lst8, and Tco89) (Cardenas and Heitman 1995; Kunz *et al.* 2000; Chen and Kaiser 2003; Wedaman *et al.* 2003; Reinke *et al.* 2004; Aronova *et al.* 2007) as well as TORC1 regulators (EGO complex) (Dubouloz *et al.* 2005; Gao and Kaiser 2006;

Kim *et al.* 2008; Binda *et al.* 2009; Bonfils *et al.* 2012) and downstream effectors, such as the Tap42–Sit4 phosphatase complex and the AGC kinase Sch9 (Yan *et al.* 2006; Urban *et al.* 2007) localize to the late endosome and vacuole membranes. Moreover, the Golgi Ca²⁺/Mn²⁺ATPase Pmr1 has been described to negatively regulate TORC1 function (Devasahayam *et al.* 2006). Finally, genetic interactions between TORC1 and representative Class C Vps proteins that mediate docking and fusion of vesicles with vacuoles have been described (Aronova *et al.* 2007; Zurita-Martinez *et al.* 2007).

Class C and D Vps proteins regulate Golgi-to-vacuole protein transport (Peterson and Emr 2001; Bowers and Stevens 2005): the Class C Vps complex, made up of Vps11/Pep5, Vps18/Pep3, Vps16, and Vps33, is required for docking and fusion at the vacuole (Rieder and Emr 1997; Srivastava *et al.* 2000; Peterson and Emr 2001; Bowers and Stevens 2005), whereas the Class D proteins (including Vps3, Vps6/Pep12, Vps34 and Vps45) are thought to control vesicular trafficking between the late Golgi and the late endosome (Prescianotto-Baschong and Riezman 2002; Bowers and Stevens 2005). Puria *et al.* (2008) have previously observed a requirement of Class C and D Vps proteins for Gln3 nuclear translocation after transferring yeast peptone dextrose (YPD) grown cells to proline medium but not after treating them with rapamycin. Aware that Gln3 and Gat1 are sometimes regulated differently, these observations prompted two obvious but important questions: (1) Did Gat1 exhibit the same responses and requirements as Gln3? (2) Would the same responses be observed if a defined, nitrogen-rich rather than complex YPD medium was employed throughout the experiments, thereby eliminating a significant variable from their interpretation?

The first outcome of our study was that Puria *et al.*'s (2008) observations are medium-specific and hence cannot be generalized. Indeed, although Gln3 nuclear localization is impaired in response to nitrogen limitation but not rapamycin treatment in YPD-pregrown *vps* mutants, we show that in yeast nitrogen base (YNB) ammonia, Vps proteins are required for Gln3 nuclear localization even in response to rapamycin. Therefore, the model describing the requirement of Vps system components for Gln3 trafficking to the nucleus (Puria and Cardenas 2008) requires modification. Second, we show that components of Golgi-to-vacuole trafficking are required for Gat1 function either in YNB-ammonia- or YPD-grown cells treated with rapamycin or transferred to proline medium. Indeed, *vps* mutations reduced the ability of Gat1 to (1) translocate to the nucleus, (2) bind to the Gat1-activated *DAL5* (encoding allantoin permease) promoter, and (3) elicit *DAL5* expression. Additionally, we show that Gat1, like Gln3, fractionates with light intracellular membranes, raising the possibility

that its regulation might occur at these locations too. Finally, our observations suggest that Ure2 might function downstream the Vps proteins during GATA factor-mediated signaling.

Experimental Procedures

Strains and culture conditions

S. cerevisiae strains used in this work are listed in Table 1. Deletion strains involving insertion of kanMX or natMX cassettes were constructed using the short and long flanking homology strategy of Wach (1996) using primers described in Table 1. Gat1-Myc¹³ and Gln3-Myc¹³ protein production was controlled in each *vps* mutant and their levels were comparable to the isogenic wild types (WT). Cultures were grown to midlog phase ($A_{600\text{nm}} = 0.5\text{--}0.55$) in YPD medium or YNB minimal medium containing ammonia at a 1% final concentration. Appropriate supplements ($100\ \mu\text{g mL}^{-1}$ leucine, $20\ \mu\text{g mL}^{-1}$ uracil, histidine, tryptophan) were added to the medium as necessary to cover auxotrophic requirements. Where indicated, cells were treated for 20 min with $200\ \text{ng mL}^{-1}$ rapamycin or transferred to YNB minimal medium containing 0.1% proline for 60 min prior to assay.

Quantitative real-time polymerase chain reaction

RNA isolation and cDNA synthesis were conducted as described in (Georis *et al.* 2009). Quantification of specific cDNA targets was measured by real-time polymerase chain reaction (PCR) performed on a StepOnePlus device (Applied Biosystems, Foster City, CA) using *DAL5* and *GAP1* primers that have been described previously (Georis *et al.* 2008, 2009). *MEP2*-specific primers are MEP2-O9 (5'-ACGAGGAATCCACTGCTTAC-3') and MEP2-O10 (5'-TTTCTGCGTCTGTGTTACCC-3'). The values reported represent the averages of at least two experiments from independent cultures; error bars indicate standard errors.

Chromatin immunoprecipitation

Cell extracts and immunoprecipitations were conducted as described in (Georis *et al.* 2008, 2009). Concentrations of specific DNA targets in immunoprecipitation (IP) and input (IN) samples were measured by real-time PCR performed on a StepOnePlus device (Applied Biosystems) using primers described in (Georis *et al.* 2008). The values reported represent the averages of two immunoprecipitations performed in at least two experiments from independent cultures; error bars indicate standard errors.

Intracellular Gln3 and Gat1 localization

Gln3-Myc¹³ and Gat1-Myc¹³ was visualized by indirect immunofluorescence of whole fixed cells. Immunofluorescence analysis was performed according to standard procedures (Pringle *et al.* 1991). Cells were fixed for 30 min in formaldehyde (3.7%). They were washed and resuspended in sorbitol buffer (1.2 mol/L sorbitol and 100 mmol/L potassium phosphate, pH 7.5). Cell walls were digested for 50 min at 30°C in sorbitol buffer supplemented with β -mercaptoethanol (40 mmol/L final) and lyticase ($80\ \text{U mL}^{-1}$) (92807 Fluka, St. Gallen, Switzerland). Gln3-Myc¹³ was detected with monoclonal antibody c-Myc (9E10) (Santa Cruz, Dallas, TX) at a working dilution of 1:30. The secondary antibody was Alexa Fluor 488 goat anti-mouse immunoglobulin G (heavy plus light chains) (A-11029; Molecular probes, Carlsbad, CA) (working dilution, 1:200). DNA was stained with DAPI (4',6'-diamidino-2-phenylindole). Cells were imaged using a fluorescence microscope (Nikon eclipse 80i; Nikon, Tokyo, Japan) equipped with digital sight DS-U3 fluorescence source. Images were captured with a digital camera (Digital sight, DS-U3) and Nikon Instrument Software elements, version 3.22 acquisition software and processed for publication with Photoshop CS (Adobe Systems, San Jose, CA).

To more representatively and completely describe images of Gln3-Myc¹³ and Gat1-Myc¹³ localization appearing in the figures of this paper, primary images were captured and saved in JPEG. We manually scored Gln3-Myc¹³ and Gat1-Myc¹³ localizations in 200 or more cells from six different microscopic fields from which these multiple primary images were taken. For scoring the unchanged images were used. For image publication, a portion of the JPEG files were processed with Photoshop CS (Adobe Systems) where changes were made only to decrease background fluorescence. Cells were classified into one of three categories: cytoplasmic (fluorescence in the cytoplasm only), nuclear–cytoplasmic (fluorescence in the cytoplasm and nucleus), and nuclear (fluorescence in the nucleus only). Although scoring limitations and reproducibility were described in Tate *et al.* (2006), we emphasize that the nuclear–cytoplasmic category is, of necessity, arbitrary. Placing cells in that category is based on subjective visual evaluation by the individual scoring the cells; it is not an objective instrument-based measurement. When the fluorescent signal is not restricted to a single cellular compartment, scoring depends upon repeated decisions of whether it is nuclear–cytoplasmic or a category flanking it. They will undoubtedly differ in detail from those of another observer, who sets their category dividing lines differently. Our intracellular distributions were scored as consistently as possible. Results were

Table 1. Strains used in this work.

Strain	Pertinent genotype	Parent	Complete genotype	Reference	Primer/Reference
TB50	WT		<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa</i>	Beck and Hall (1999)	
TB123	W.T. Gln3-Myc ¹³		<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa, GLN3-MYC¹³[KanMX]</i>	Beck and Hall (1999)	
JK9-3d	W.T.		<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa</i>	Beck and Hall (1999)	
MK23	<i>vps3Δ</i>	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, vps3::kanMX</i>	This work	<i>vps3</i> : 5', -42 to -1 and 3', 3037 to 3076
MK24	<i>pep5Δ</i>	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa, pep5::kanMX</i>	This work	<i>pep5</i> : 5', -41 to -1 and 3', 3091 to 3133
MK27	<i>vps3Δ</i> Gln3-Myc ¹³	FV250	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, vps3::kanMX GLN3-MYC¹³[HIS3]</i>	This work	<i>vps3</i> : 5', -42 to -1 and 3', 3037 to 3076
MK30	<i>pep5Δ</i> Gln3-Myc ¹³	FV250	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, pep5::kanMX GLN3-MYC¹³[HIS3]</i>	This work	<i>pep5</i> : 5', -41 to -1 and 3', 3091 to 3133
MK46	<i>pep3Δ</i>	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, pep3::natMX</i>	This work	<i>pep3</i> : 5', -42 to -1 and 3', 2758 to 2801
08047c	<i>ure2Δvps3Δ</i>	OK01 X MK23	<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa, ure2::natMX, vps3::kanMX</i>	This work	
OK01	<i>ure2Δ</i>	JK9-3d	<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa, ure2::natMX</i>	This work	Ure2-L1, Ure2-L2, Ure2-L3 and Ure2-L4 (Georis et al. 2008)
FV005	<i>gln3Δ</i>	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, gln3::kanMX</i>	Georis et al. (2008)	
FV006	<i>gat1Δ</i>	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, gat1::kanMX</i>	Georis et al. (2008)	
FV018	<i>gat1Δ</i> Gln3-Myc ¹³	TB123	<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa, gat1::natMX GLN3-MYC¹³[KanMX]</i>	Georis et al. (2008)	
FV063	W.T. Gat1-Myc ¹³	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GAT1-MYC¹³[HIS3]</i>	Georis et al. (2008)	
FV064	<i>gln3Δ</i> Gat1-Myc ¹³	FV005	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, gln3::kanMX GAT1-MYC¹³[HIS3]</i>	Georis et al. (2008)	
FV250	W.T. Gln3-Myc ¹³	TB50	<i>MATa, leu2-3, 112, ura3-52, trp1, his4, rme1, HMLa, GLN3-MYC¹³[HIS3]</i>	Georis et al. (2011b)	
FV390	<i>vps34Δ</i> Gln3-Myc ¹³	TB123	<i>MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, vps34::natMX, GLN3-MYC¹³[KanMX]</i>	This work	<i>vps34</i> : 5', -430 to -407 and -1 to -33; 3', 2629 to 2664 and 3152 to 3171

Table 1. (Continued).

Strain	Pertinent genotype	Parent	Complete genotype	Reference	Primer/Reference
FV391	<i>vps34Δ</i>	TB50	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps34::kanMX</i>	This work	<i>vps34</i> : 5', -430 to -407 and -1 to -33; 3', 2629 to 2664 and 3152 to 3171
FV392	<i>vps34Δ Gat1-Myc¹³</i>	FV063	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps34::kanMX GAT1-MYC¹³[HIS3]</i>	This work	<i>vps34</i> : 5', -430 to -407 and -1 to -33; 3', 2629 to 2664 and 3152 to 3171
FV640	<i>pep5Δ Gat1-Myc¹³</i>	FV063	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, pep5::kanMX GAT1-MYC¹³[HIS3]</i>	This work	<i>pep5</i> : 5', -41 to -1 and 3', 3091 to 3133
FV641	<i>vps3Δ Gat1-Myc¹³</i>	FV063	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps3::kanMX GAT1-MYC¹³[HIS3]</i>	This work	<i>vps3</i> : 5', -42 to -1 and 3', 3037 to 3076
FV642	<i>vps45Δ Gat1-Myc¹³</i>	FV063	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps45::kanMX GAT1-MYC¹³[HIS3]</i>	This work	<i>vps45</i> : 5', -42 to -1 and 3', 1735 to 1778
FV643	<i>vps45Δ</i>	TB50	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps45::kanMX</i>	This work	<i>vps45</i> : 5', -42 to -1 and 3', 1735 to 1778
FV644	<i>vps45Δ Gln3-Myc¹³</i>	FV250	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps45::kanMX GLN3-MYC¹³[HIS3]</i>	This work	<i>vps45</i> : 5', -42 to -1 and 3', 1735 to 1778
FV732	<i>pep3Δ Gat1-Myc¹³</i>	FV063	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, pep3::kanMX GAT1-MYC¹³[HIS3]</i>	This work	<i>pep3</i> : 5', -42 to -1 and 3', 2758 to 2801
FV733	<i>pep3Δ Gln3-Myc¹³</i>	FV250	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, pep3::kanMX GLN3-MYC¹³[HIS3]</i>	This work	<i>pep3</i> : 5', -42 to -1 and 3', 2758 to 2801
FV734	<i>vps16Δ Gat1-Myc¹³</i>	FV063	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps16::kanMX GAT1-MYC¹³[HIS3]</i>	This work	<i>vps16</i> : 5', -42 to -1 and 3', 2397 to 2439
FV735	<i>vps16Δ</i>	TB50	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps16::kanMX</i>	This work	<i>vps16</i> : 5', -42 to -1 and 3', 2397 to 2439
FV736	<i>vps16Δ Gln3-Myc¹³</i>	FV250	<i>MATa, leu2-3,112, ura3-52, trp1, his3, rme1, HMLa, vps16::kanMX GLN3-MYC¹³[HIS3]</i>	This work	<i>vps16</i> : 5', -42 to -1 and 3', 2397 to 2439

confirmed using independent cultures. Detailed examples of the three scoring categories for Gln3-Myc¹³ and Gat1-Myc¹³ as well as scoring precision (within and between experiments) appear in references (Tate et al. 2006, 2009, 2010; Tate and Cooper 2007, 2008; Georis et al. 2008).

Subcellular fractionation

Cells were lysed in buffer A containing 50 mmol/L Tris (pH 7.5), 0.2 mol/L sorbitol, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol (DTT) or buffer B containing 0.1 mol/L Tris (pH 7.5), 0.15 mol/L

NaCl, 5 mmol/L EDTA. Protease and phosphatase inhibitor cocktails were added for whole-cell extract preparation. Unbroken cells were removed by centrifugation at 500g for 10 min. The cell-free extract was centrifuged at 13,000g for 15 min to yield P13 (pellet) fraction. The supernatant was then centrifuged at 100,000g for 40 min to obtain P100 (pellet) and S100 (supernatant). The different subcellular fractions were analyzed by Western blotting with monoclonal antibodies for c-Myc (Santa Cruz Biotechnology), Pep12 (Invitrogen) and Pkg1 (Invitrogen, Carlsbad, CA). The electrophoresis, blotting, and detection procedures have been described previously (Georis et al. 2009).

Results

Class C and D *vps* mutants are defective for activating *DAL5* expression in response to rapamycin or transferring cells from ammonia to proline medium

In order to examine the requirement of proteins that participate in Golgi-to-vacuole trafficking for the control of NCR-sensitive gene expression, we assayed *DAL5* expression in YNB-ammonia-grown untreated, rapamycin-treated or proline-transferred WT, *gln3Δ* and *gat1Δ* as well as Class C (*pep3Δ*, *pep5Δ* and *vps16Δ*) and D (*vps3Δ*, *vps34Δ* and *vps45Δ*) *vps* mutant cells (Fig. 1). The expression levels exhibited by rapamycin-treated or proline-transferred WT cells were clearly impaired in *gln3Δ* or *gat1Δ*-mutant cells, demonstrating that *DAL5* transcription requires the simultaneous presence of Gln3 and Gat1 when YNB-ammonia-grown cells are transferred to proline medium,

or treated with rapamycin (Georis et al. 2008). After a transfer to proline, *DAL5* expression was lost in all *vps* mutant cells compared with a WT strain, whereas residual expression still occurred after rapamycin treatment (Fig. 1). Impaired *DAL5* expression in rapamycin-treated or *vps* mutant cells transferred to proline could be attributed to loss of Gln3 function alone, Gat1 alone or the functions of both Gln3 and Gat1 together. Importantly, *DAL5* expression decreased more when *vps* mutants were transferred from ammonia to proline medium than in either the *gln3Δ* or *gat1Δ* strains, suggesting that components of the Golgi-to-vacuole trafficking system may be required for both Gat1 and Gln3 function.

Components of Golgi-to-vacuole trafficking are required for efficient GATA factor binding to the Gat1-activated *DAL5* promoter

To test whether the observed requirement of representative Class C and D Vps proteins for *DAL5* expression following rapamycin treatment or transfer to proline medium affected GATA factor binding to the *DAL5* promoter, chromatin immunoprecipitation experiments were performed in the strains described above. Gat1-dependent Gln3-Myc¹³ binding to the *DAL5* promoter was elicited in rapamycin-treated or proline-transferred WT cells (Fig. 2) (Georis et al. 2008). Cells lacking Gat1 or any of the Vps proteins tested displayed an impaired Gln3-Myc¹³ binding in both rapamycin-treated and proline-transferred cultures (Fig. 2). However, some residual binding did remain in the rapamycin-treated

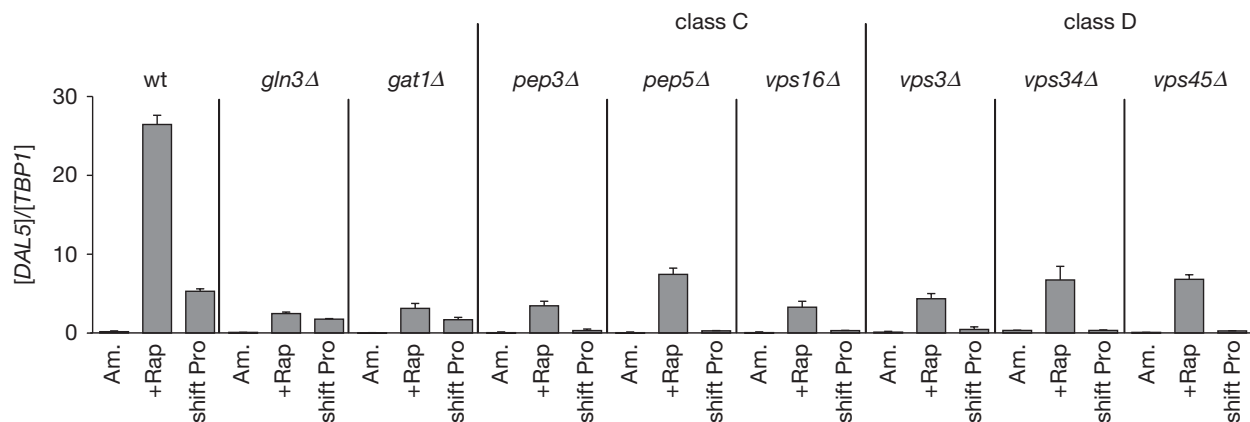


Figure 1. Class C and D Vps proteins requirements for efficient transcription of the Gat1-activated *DAL5* gene following rapamycin treatment or transferring cells from ammonia to proline medium in YNB-grown cells. Total RNA was isolated from WT (TB50), *gln3Δ* (FV005), *gat1Δ* (FV006), the Class C *pep3Δ* (MK46), *pep5Δ* (MK24), and *vps16Δ* (FV735) and the Class D *vps3Δ* (MK23), *vps34Δ* (FV391), and *vps45Δ* (FV643) mutant cells grown in YNB-ammonia medium (Am.) and treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). *DAL5* mRNA levels were quantified by quantitative RT-PCR as described in "Experimental Procedures." The values reported represent the averages of at least two experiments from independent cultures; error bars indicate standard errors.

cells. Gat1-Myc¹³ was efficiently recruited to the *DAL5* promoter in rapamycin-treated WT cells (Georis *et al.* 2008), but only about half as efficiently following transfer to proline medium (Fig. 2). Gat1 binding in cells transferred to proline medium required Gln3 and all of the Vps components tested. In contrast, high level Gat1-Myc¹³ binding to the *DAL5* promoter following rapamycin treatment required only the Vps proteins assayed but not Gln3. This observation demonstrates that Gat1 binding in rapamycin-treated cells largely requires functional Golgi-to-vacuole trafficking components, independent of Gln3 function. On the other hand, impaired Gln3 binding observed in rapamycin-treated or proline-transferred *vps* mutants, could be either due to a direct requirement of the Golgi-to-vacuole trafficking component proteins we assayed or as a consequence of impaired Gat1 binding.

Efficient nuclear GATA factor localization in rapamycin-treated or proline-transferred cells requires Class C and D Vps proteins

To determine whether impaired GATA factor binding to DNA derived from impaired nuclear localization in the *vps* mutants, we characterized Gat1-Myc¹³ and Gln3-Myc¹³ localization using indirect immunofluorescence. Gat1-Myc¹³ nuclear localization in rapamycin-treated or proline-transferred cells pregrown in YNB-ammonia was reduced in the Class C and D *vps* mutants relative to WT (Fig. 3). Following rapamycin treatment, Gat1-Myc¹³ was mainly nuclear–cytoplasmic in all tested *vps* mutant cells rather than fully nuclear as occurred in WT cells. In cells transferred to proline medium, the fraction of *pep3Δ*, *pep5Δ*, *vps16Δ*, *vps3Δ*, *vps34Δ*, *vps45Δ* cells where Gat1-Myc¹³ was nuclear or nuclear–cytoplasmic

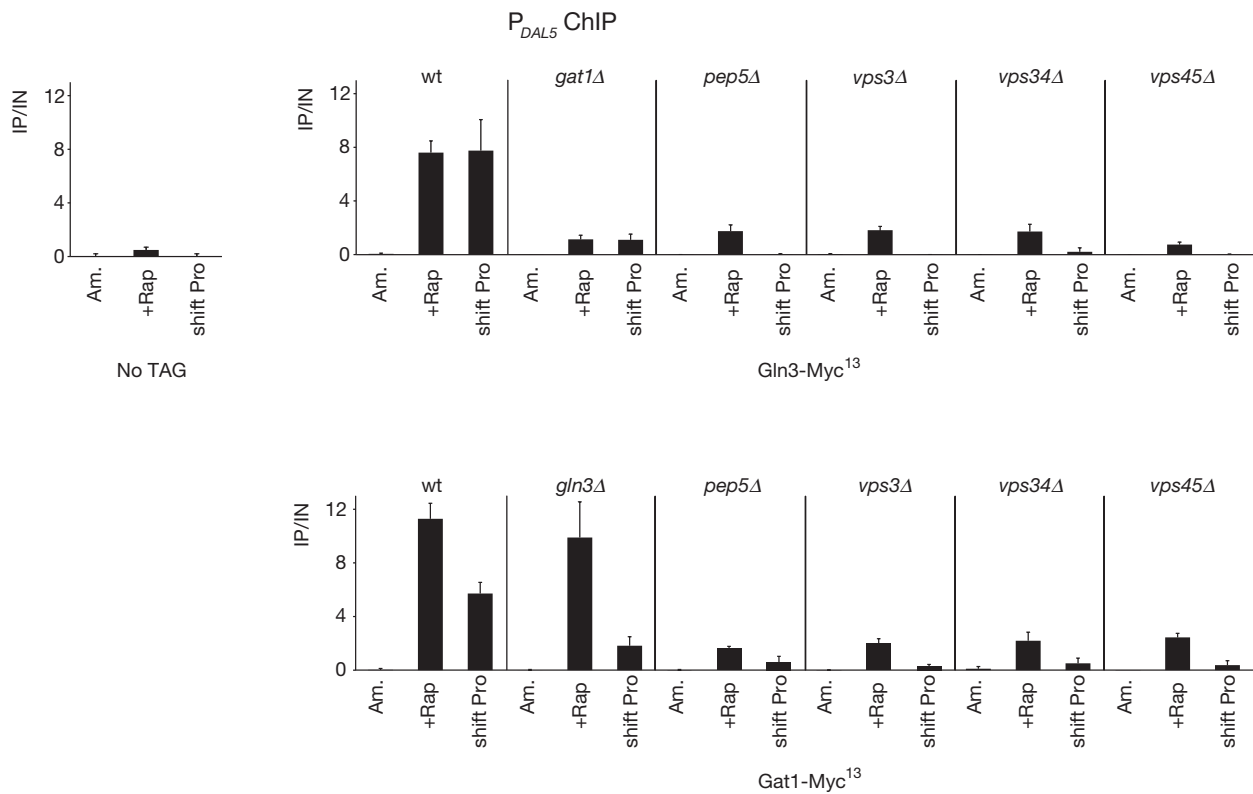


Figure 2. Gln3-Myc¹³ and Gat1-Myc¹³ binding to the *DAL5* promoter in WT, *gln3Δ* and *gat1Δ* cells as well as in the Class C *pep5Δ* mutant and the Class D *vps3Δ*, *vps34Δ*, and *vps45Δ* mutant cells in response to rapamycin treatment or transferring cells from YNB-ammonia to YNB-proline medium. Untagged WT (TB50), *GLN3-MYC¹³* WT (FV250), *GLN3-MYC¹³ gat1Δ* (FV018), *GLN3-MYC¹³ pep5Δ* (MK30), *GLN3-MYC¹³ vps3Δ* (MK27), *GLN3-MYC¹³ vps34Δ* (FV390), *GLN3-MYC¹³ vps45Δ* (FV644), *GAT1-MYC¹³* WT (FV063), *GAT1-MYC¹³ gln3Δ* (FV064), *GAT1-MYC¹³ pep5Δ* (FV640), *GAT1-MYC¹³ vps3Δ* (FV641), *GAT1-MYC¹³ vps34Δ* (FV392), and *GAT1-MYC¹³ vps45Δ* (FV642) cells were grown in YNB-ammonia medium (Am.) and treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). ChIP was performed using antibodies against c-myc as described in “Experimental Procedures.” qPCR of IP and IN fractions was performed with primers specific for the *DAL5* promoter (*DAL5P*) and for a region 2.5 kb upstream of the *DAL5* open reading frame as a control (*DAL5U*). For each immunoprecipitation, IP/IN values were calculated as follows: ($[DAL5P]^{IP}/[DAL5P]^{IN} - [DAL5U]^{IP}/[DAL5U]^{IN}$). The values reported represent the averages of two immunoprecipitations performed in at least two experiments from independent cultures; error bars indicate standard errors.

also decreased, accompanied by increased cytoplasmic Gat1-Myc¹³ localization. In none of the cases, however, were the Vps protein requirements absolute. These observations indicate that Class C and D Vps proteins are partially required for efficient Gat1 translocation to the nucleus. In contrast, Class C and D *vps* mutations totally abolished Gln3-Myc¹³ nuclear localization not only in cells transferred to proline medium but also, surprisingly, after rapamycin treatment (Fig. 4). These observations, obtained with YNB medium, contrast with a previous report showing that Golgi-to-vacuole trafficking components were dispensable for rapamycin-elicited Gln3 nuclear translocation (Puria *et al.* 2008).

A media-dependent role of Class C and D Vps proteins for Gln3 and Gat1 nuclear translocation and *DAL5* gene activation

To test if the apparent discrepancy between present and previously reported results was due to a technical problem or the different growth conditions employed, we characterized Gln3-Myc¹³ localization in WT, *pep5Δ* (chosen as Class C representative) and *vps34Δ* (chosen as Class D representative) mutant cells using the same growth conditions as Puria *et al.* (2008), that is, in YPD medium (untreated, rapamycin-treated or after transfer to proline medium). Our observations (Fig. 5A) were in agreement

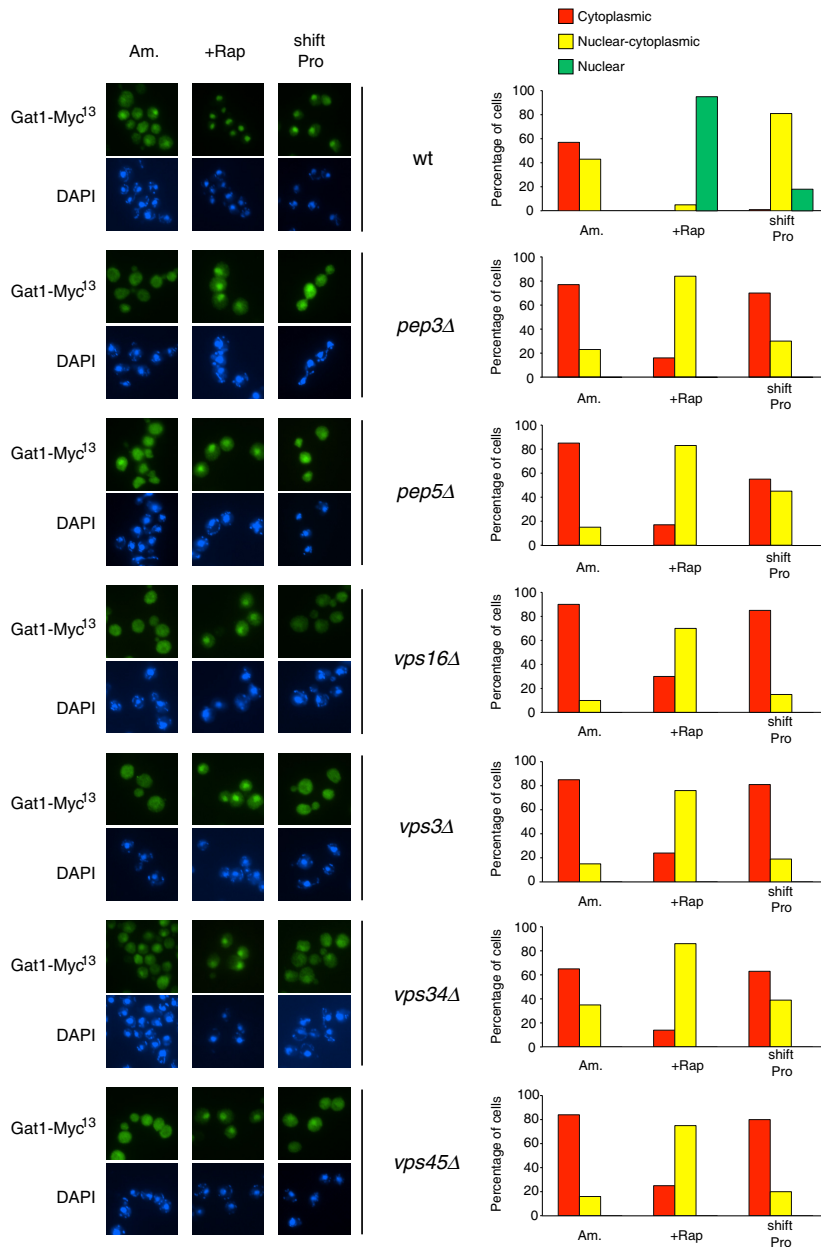


Figure 3. Requirements of Class C and D Vps proteins for intracellular Gat1-Myc¹³ localization in response to rapamycin or transferring cells from YNB-ammonia to YNB-proline medium. *GAT1-MYC¹³* WT (FV063), Class C *pep3Δ* (FV732), *pep5Δ* (FV640), and *vps16Δ* (FV734) and Class D *vps3Δ* (FV641), *vps34Δ* (FV392), and *vps45Δ* (FV642) mutant cells were grown in YNB-ammonia medium (Am.). Cells were treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). The cultures were sampled for indirect immunofluorescence assay of Gat1-Myc¹³ localization. Indirect immunofluorescence assays were performed and imaged as described in Experimental Procedures. Images from which the histograms were derived are displayed on the left hand side of the figure. The upper member of each pair depicts green Gat1-Myc¹³-derived fluorescence and the lower one shows DAPI-positive material fluorescence. For each histogram, displayed at the right hand side of the figure, cells were scored for intracellular Gat1-Myc¹³ localization (cytoplasmic red bars, nuclear-cytoplasmic, yellow bars; nuclear, green bars) using criteria described in Experimental Procedures. When no histogram bar is visible on the graph, that is because there were no cells found in scoring category considered. The values reported represent the averages of at least two experiments from independent cultures.

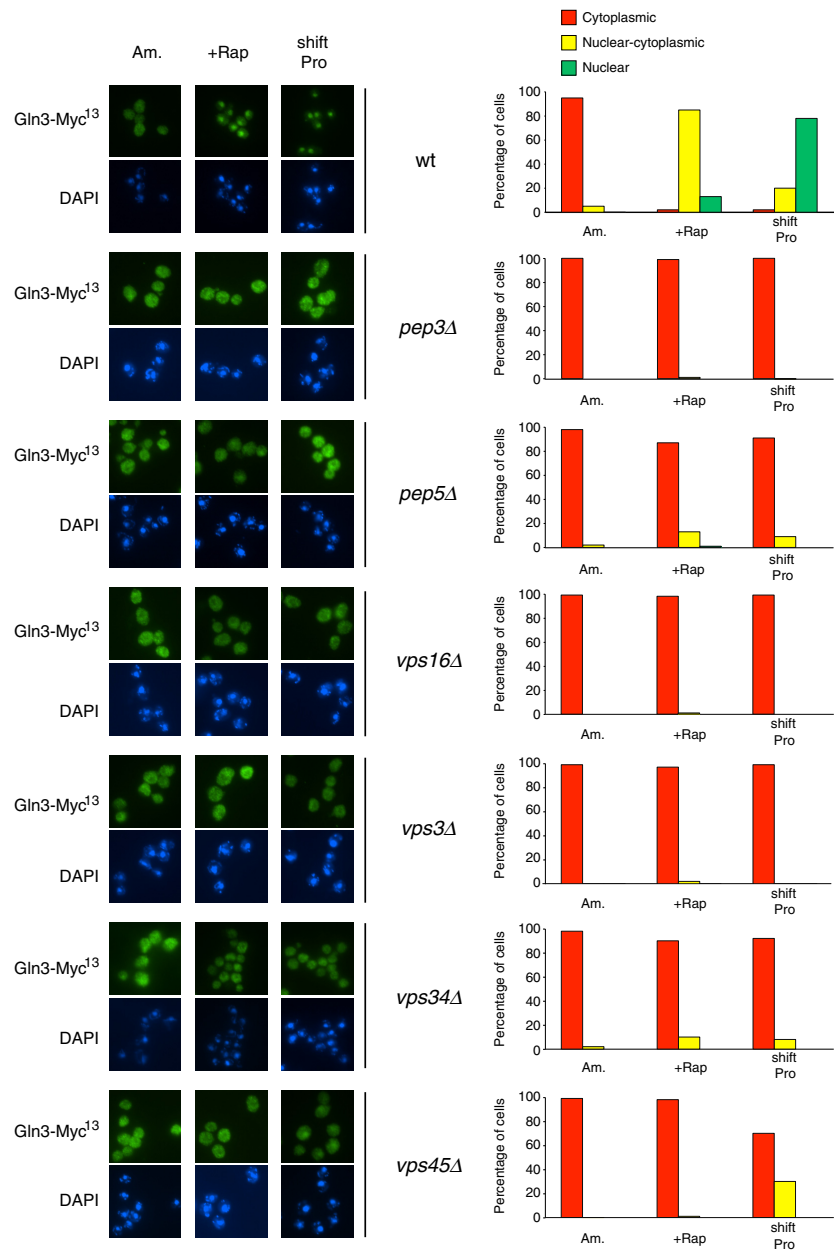


Figure 4. Requirements of Class C and D Vps proteins for intracellular Gln3-Myc¹³ localization in response to rapamycin or transferring cells from YNB-ammonia to YNB-proline medium. *GLN3-MYC¹³* WT (FV250), Class C *pep3Δ* (FV733), *pep5Δ* (MK30), and *vps16Δ* (FV736) and Class D *vps3Δ* (MK27), *vps34Δ* (FV390), and *vps45Δ* (FV644) mutant cells were grown in YNB-ammonia medium (Am.) and treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). The experimental format and data presentation are the same as those in Figure 3.

with previous studies showing that mutations in Class C and D *VPS* genes impair Gln3 nuclear localization following a transfer to proline medium but not to rapamycin treatment (Puria *et al.* 2008), indicating that the observed discrepancy was indeed due to the nature of the growth medium.

The same procedure was followed to assess Gat1-Myc¹³ localization. As occurred in YNB-ammonia-grown WT cells (Fig. 3), rapamycin addition to YPD-grown cells led to a fully nuclear localization of Gat1-Myc¹³ (Fig. 5B). Deletion of *VPS34* or *PEP5* in rapamycin-treated cells reduced Gat1-Myc¹³ nuclear localization by increasing the

fraction of cells in which Gat1-Myc¹³ was nuclear–cytoplasmic at the expense of cells where Gat1-Myc¹³ was fully nuclear. Gat1-Myc¹³ nuclear localization in response to transferring cells to proline medium was also partially impaired in the *vps34Δ* and *pep5Δ* mutants relative to WT. This was characterized by an increase in the fraction of cells where Gat1-Myc¹³ was cytoplasmic and a decrease in the fraction of cells where Gat1-Myc¹³ was nuclear and/or nuclear–cytoplasmic.

To test whether the observed requirement of Class C and D Vps proteins for Gat1-Myc¹³ nuclear localization upon rapamycin treatment or transferring cells to proline

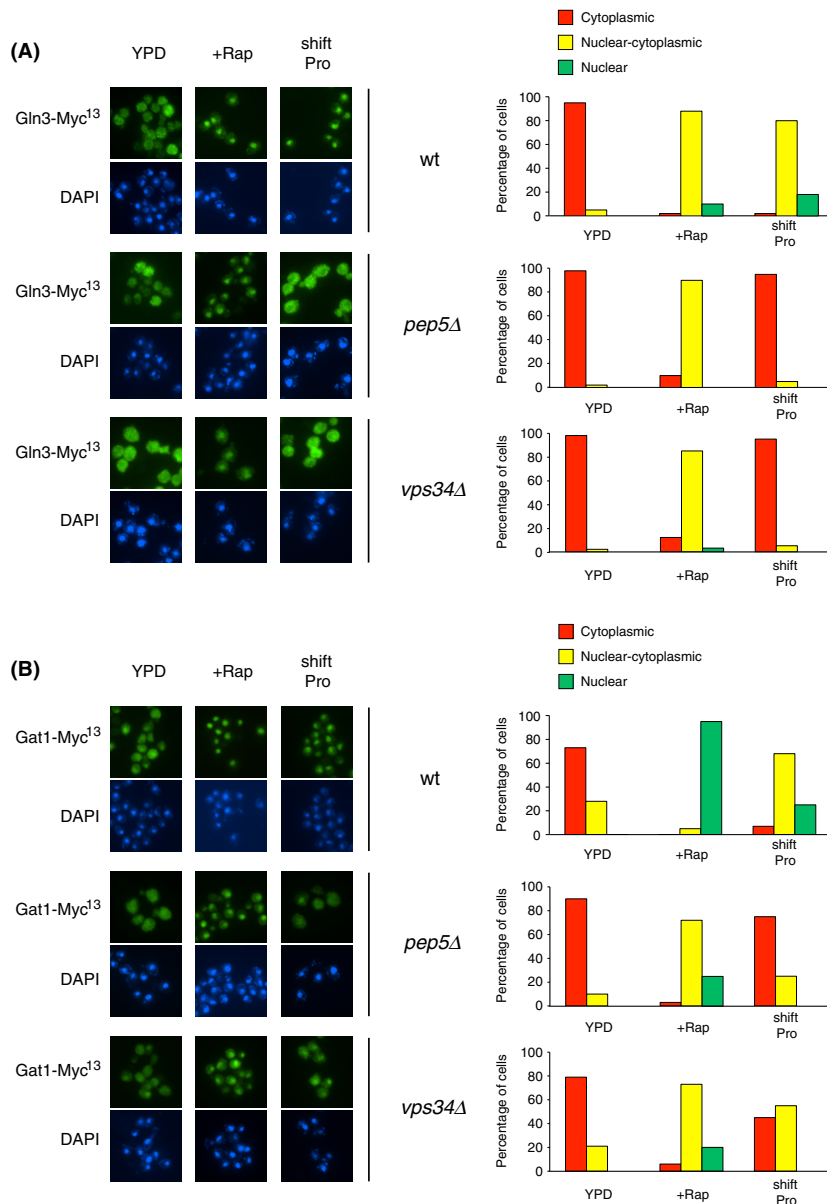


Figure 5. Requirements of Class C and D Vps proteins for intracellular Gln3-Myc¹³ and Gat1-Myc¹³ localization in YPD growth conditions. *GLN3-MYC¹³* WT (FV250), *pep5Δ* (MK30), *vps34Δ* (FV390), *GAT1-MYC¹³* WT (FV063), *pep5Δ* (FV640), and *vps34Δ* (FV392) cells were grown in YPD medium and treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). The experimental format and data presentation are the same as those in Figure 3 with one exception that all the strains were grown in YPD medium instead of YNB-ammonia.

medium paralleled its ability to bind to the *DAL5* promoter, chromatin immunoprecipitation experiments were performed in WT and *vps34Δ* cells (Fig. 6). Much more Gat1-Myc¹³ was recruited to the *DAL5* promoter in rapamycin-treated WT cells compared with untreated YPD-grown cells. In contrast, Gat1-Myc¹³ binding to the *DAL5* promoter in *vps34Δ* cells treated with rapamycin or transferred to proline medium was reduced.

Finally, we determined the transcription profiles of *DAL5*, *GAP1*, and *MEP2* genes in YPD-grown, untreated, rapamycin-treated, or proline-transferred WT, *gln3Δ*, *gat1Δ* and Class C and D *vps* mutant cells (Fig. 7). The *DAL5* expression levels elicited by rapamycin-treated or

proline-transferred WT cells were clearly decreased in *gln3Δ* or *gat1Δ* mutant cells, demonstrating that, as occurred in YNB medium, *DAL5* transcription requires both GATA factors under these conditions (Fig. 7A). After rapamycin treatment or transfer to proline medium, *DAL5* expression was clearly reduced in all *vps* mutants relative to WT cells (Fig. 7A). As Gln3-Myc¹³ nuclear localization is largely intact, impaired *DAL5* expression in rapamycin-treated *vps* mutant cells should result from the reduced Gat1-Myc¹³ nuclear localization and DNA binding. On the other hand, the impaired *DAL5* expression exhibited by the *vps* mutants in response to transferring cells into proline medium could be attributed to the impaired nuclear

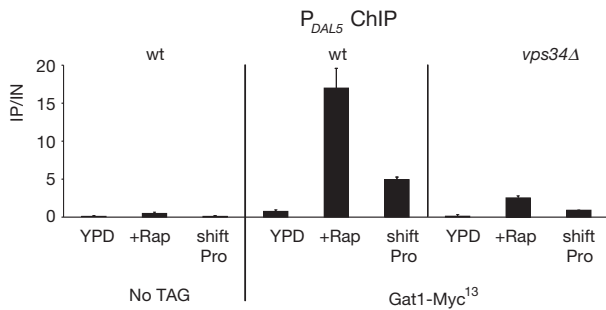


Figure 6. Gat1-Myc¹³ binding to the *DAL5* promoter in WT and *vps34Δ* strains in response to rapamycin or transfer of YPD-grown cells to YNB-proline medium. Untagged WT (TB50), *GAT1-MYC¹³* WT (FV063) and *GAT1-MYC¹³ vps34Δ* (FV392) cells were grown in YPD medium and treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). ChIP was performed as described in Figure 2.

localization of either Gln3-Myc¹³, Gat1-Myc¹³ or of both GATA activators.

GAP1 or *MEP2* expression levels in YPD-grown WT cells treated with rapamycin or transferred to proline medium were only slightly reduced in *gln3Δ* or *gat1Δ* mutant cells, demonstrating that either Gln3 or Gat1 alone could sustain *GAP1* or *MEP2* expression in rapamycin-treated cells or in cells transferred to proline medium (Fig. 7B and C). Consistent with previous data (Puria et al. 2008), only in cells transferred to proline medium but not those treated with rapamycin, were *GAP1* and *MEP2* expression levels reduced in all *vps* mutant cells compared with a WT strain (Fig. 7B and C). The unaffected expression observed after rapamycin treatment in the *vps* mutants correlated with the intact nuclear translocation of Gln3. On the other hand, impaired expression observed in *vps* mutant cells transferred to proline medium was in agreement with the impaired nuclear translocation of both Gln3 and Gat1.

A fraction of Gat1 associates with light intracellular membranes

Several regulators of GATA factor-activated genes, for example, TORC1, Tap42-Sit4 phosphatase, as well as Gln3 have been shown to be associated with intracellular light membranes (Cardenas and Heitman 1995; Kunz et al. 2000; Chen and Kaiser 2003; Wedaman et al. 2003; Reinke et al. 2004; Yan et al. 2006; Aronova et al. 2007; Puria et al. 2008). These findings, and our observations that *vps* mutant cells exhibit defects in Gat1 localization and function, raised the possibility that it too might be associated with light membranes. Therefore, Gat1-Myc¹³ subcellular fractionation was performed to assess this possibility (Fig. 8A). To evaluate the degree to which our procedure cleanly separated soluble from insoluble proteins, we examined the distribution of the cytosolic marker Pgk1 and the

membrane-associated protein, Pep12. As expected, Pgk1 was recovered primarily in the supernatant fraction, whereas the endosomal marker Pep12 was distributed between both the P13 and P100 fractions (Fig. 8A), as described previously (Becherer et al. 1996). Gat1-Myc¹³ was detected as a typical double band, corresponding to two isoforms possessing different N-termini (Rai et al. 2013a). In WT cells, Gat1-Myc¹³, like Gln3-Myc¹³, was fractionated not only with heavy (P13) membranes (Fig. 8A for Gat1-Myc¹³ and B for Gln3-Myc¹³) known to contain plasma and endoplasmic reticulum membranes but also with lighter (P100) membranes containing Golgi and the endosomal marker Pep12. In contrast with membrane-associated Pep12, significant portions of Gat1-Myc¹³ and Gln3-Myc¹³ were still detected in the S13 and S100 fractions along with the cytosolic marker Pgk1, indicating that only a portion of Gat1-Myc¹³ and Gln3-Myc¹³ were associated with intracellular membranes.

Gln3 association with intracellular membranes has already been reported as likely being peripheral as indicated by its sensitivity to extraction with NaCl (Puria et al. 2008). To test this characteristic for Gat1, protein extracts were prepared using a stringent lysis buffer (containing 0.15 mol/L NaCl). Under such stringent conditions, Gat1-Myc¹³ fractionated largely with heavy (P13) membranes (Fig. 8C) but only residually with lighter (P100) ones. Interestingly, the two isoforms of Gat1-Myc¹³ were unevenly distributed between the supernatant and the pellet fractions. The faster migrating isoform, beginning at Gat1 methionines 95 or 102 (Rai et al. 2013a), fractionated mainly in the soluble fractions, whereas the slower migrating isoform, beginning at Gat1 methionine 40 (Rai et al. 2013a), was mainly associated with the P13 fraction. As expected, Gln3-Myc¹³ was not detected in the pellet fraction under these stringent conditions (Fig. 8D). These findings indicate that Gat1 appears to be more stably associated with light membranes than Gln3.

Ure2 seems to act downstream of Vps3 to control *DAL5* expression

Ure2 is a well-known negative regulator of GATA factor-mediated gene expression (Grenson et al. 1974; Courchesne and Magasanik 1988). Aiming at determining the epistatic relation between the *ure2Δ* and *vps* mutations, all attempts to delete *URE2* in *vps34Δ*, *pep5Δ*, or *vps45Δ* mutant cells failed, suggesting that combining the *ure2Δ* with either *vps34Δ*, *pep5Δ*, or *vps45Δ* mutations might be resulting in synthetic lethality. To test this possibility, tetrad analysis was conducted in crosses between the *vps34Δ*, *pep5Δ*, and *vps45Δ* and *ure2Δ* strains. Haploid meiotic progeny generated by sporulation of heterozygous diploids containing the above mutations carried only one mutation. No meiotic segregants carrying a double mutation were recov-

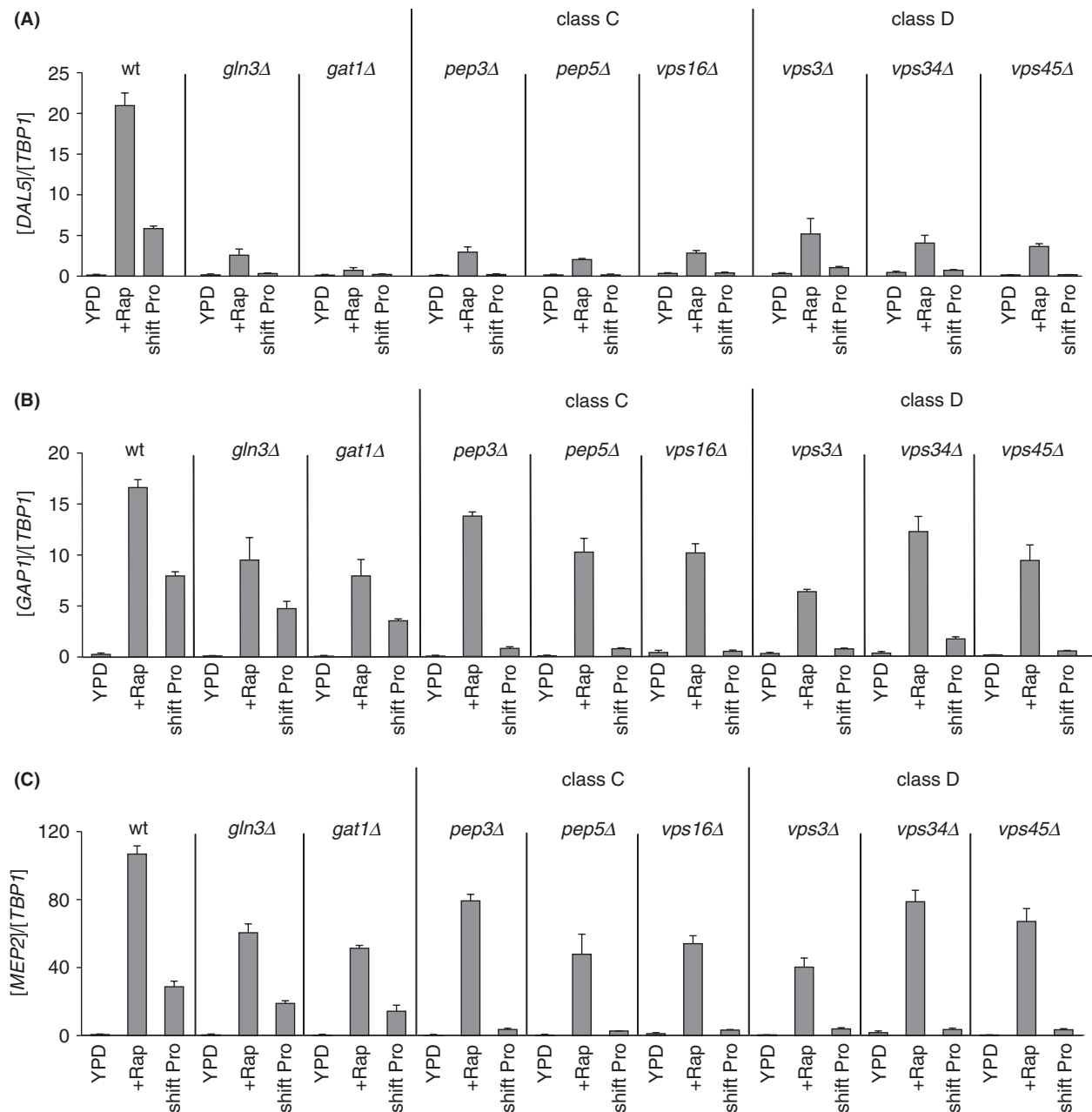


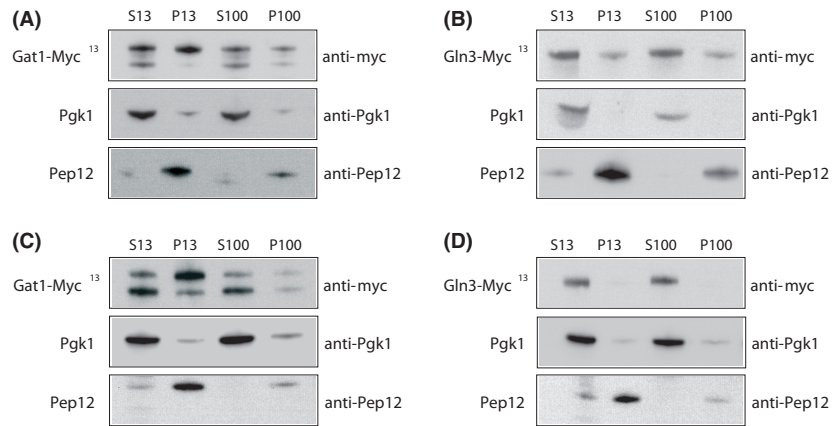
Figure 7. Class C and D Vps protein requirements for transcription of the GATA factor-activated genes *DAL5*, *GAP1* and *MEP2* in response to rapamycin or transfer of YPD-grown cells to YNB-proline medium. The strains, experimental format and data presentation are the same as those in Figure 1 with one exception that all the strains were grown in YPD medium instead of YNB-ammonia. (A) *DAL5*. (B) *GAP1*. (C) *MEP2*.

ered, confirming that these double mutants were synthetically lethal. Double *ure2Δvps3Δ* mutants, however, were recovered on YPD medium following sporulation of a *VPS3ure2Δ/vps3ΔURE2* heterozygous diploid strain, but these were unable to grow in YNB-ammonia medium.

DAL5 expression was assayed in YPD-grown untreated, rapamycin-treated, or proline-transferred WT, *ure2Δ*, *vps3Δ*, and *ure2Δvps3Δ* cells (Fig. 9). In YPD-grown cells, high-

level *DAL5* expression observed in a *ure2Δ* was lost upon additionally deleting *VPS3*. However, in response to rapamycin treatment or a transfer to proline, the elevated *DAL5* expression levels observed in *ure2Δ* were only lowered, but not lost in the double mutant. In fact, the double mutant exhibited a phenotype resembling the WT. Altogether, these observations suggest that the *vps3Δ* and *ure2Δ* mutations more likely only compensate the effects of one another.

Figure 8. Subcellular fractionation of Gat1-Myc¹³ and Gln3-Myc¹³. Cell-free lysates from YPD-grown *GAT1-MYC¹³* WT (FV063; A and C) and *GLN3-MYC¹³* WT (FV250; B and D) cells were subjected to differential centrifugation to yield low-speed pellet (P13), supernatant (S13), high-speed pellet (P100), and soluble (S100) fractions. Equal cell equivalents were examined by Western blot to detect Gln3-Myc¹³, Gat1-Myc¹³, Pep12, and Pgk1. Protein extracts were prepared using a lysis buffer lacking NaCl (A and B) or containing 0.15 mol/L NaCl (C and D).



Discussion

In this report, we show that mutations in Class C and D Vps proteins led to defects in *DAL5* expression, after treating cells with rapamycin or transferring them to YNB-proline medium. These defects correlated with altered Gat1 and Gln3 nuclear localization and DNA binding. Vps protein requirements for Gat1 localization and function were observed in cells grown either in defined, nitrogen-rich or complex YPD medium, whereas the requirements of representative Vps proteins for Gln3 function were media-specific: a requirement after rapamycin treatment was observed in YNB and not YPD medium. The results in YPD, but not YNB medium were in agreement with Puria et al.'s (2008) initial conclusions. However, the media-dependence of the Vps protein requirements for the rapamycin control of Gln3 localization and function suggests that the previously published model describing Vps protein participation in GATA factor regulation may not be correct and hence in need of revision (Puria and Cardenas 2008).

The influence of medium composition (YPD vs. YNB) on the Vps requirement for the control of Gln3 localization may not be too surprising. A previous report has indicated

that key components of the medium, such as zinc or the pH of the medium, can influence Gln3 cellular localization and GATA factor responses (Feller et al. 2006). Several differences exist between YPD and YNB media, including the pH and the quality of the nitrogen sources provided. Our YNB cultures started at a pH of 5 and were harvested at a pH of 2.7, whereas in YPD, starting and ending pHs were 6 and 4.8, respectively. Another major difference between the YPD and YNB-ammonia media is the quality of the nitrogen source, influencing the yeasts' growth rate (faster on YPD than on YNB). A third major difference is the availability of all amino acids in YPD medium, whereas YNB-ammonia-grown cells need to synthesize their own amino acids (Magasanik and Kaiser 2002; Ljungdahl and Daignan-Fornier 2012). Although it is still not obvious if and how the Vps proteins may be connected to the presence of external amino acids, we speculate that our data do not exclude the possibility that Vps proteins could be required for Gln3 relocation to the nucleus at multiple steps other than only those associated with protein trafficking to the vacuole. In mammalian cells, it has been reported that Vps34 is required for amino acids sensing and transmitting the stimulatory effects of amino acids to the TOR pathway (Byfield et al. 2005; Nobukuni et al. 2005; Backer 2008). Moreover,

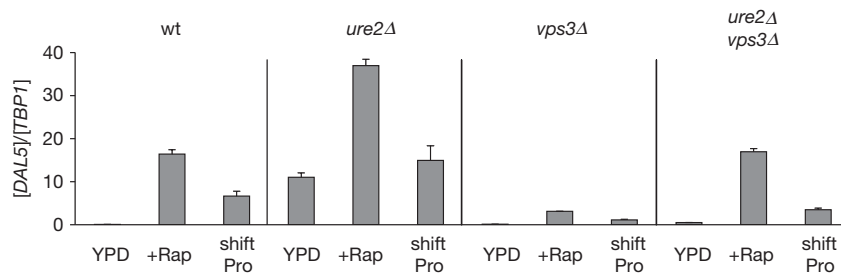


Figure 9. Epistatic Relation between Ure2 and Vps3. WT (TB50), *ure2Δ* (OK01), *vps3Δ* (MK23), *ure2Δvps3Δ* (O8047c) cells were grown in YPD medium and treated with rapamycin (+Rap) or transferred to proline medium (shift Pro). *DAL5* mRNA levels were quantified by quantitative RT-PCR as described in "Experimental Procedures." The experimental format and data presentation are the same as those in Figure 1.

and in agreement with their multiple functions, two distinct Vps34 complexes function in autophagy and carboxypeptidase Y sorting in response to nitrogen starvation (Kihara et al. 2001).

In addition to the requirement of normal Golgi-to-vacuole trafficking components for Gln3 and Gat1 nuclear localization, we show that Gat1, like Gln3 (Puria et al. 2008), associates with light membranes probably derived from the Golgi apparatus, although the former seems to be more stably associated with light membranes than the latter. Interestingly, the two Gat1 isoforms appeared to behave differently: irrespective of the lysis conditions, the faster migrating form was always observed in the cytosolic fraction, whereas the slower migrating form was more associated with the membrane fraction. It is possible that the 55 N-terminal amino acids lacking in the shorter isoform (Rai et al. 2013a) are determinants of interactions leading to membrane association. In line with this hypothesis, mutants affecting the N-terminal methionines display altered cytoplasmic retention in repressing conditions although this did not affect Gat1's transactivation capacities (Rai et al. 2013a).

Our results also suggest that Ure2 most likely participates in GATA factor regulation downstream the Vps proteins. Aware that genetic interactions exist between *URE2* and several *VPS* genes (Costanzo et al. 2010; Hoppins et al. 2011), the synthetic lethality exhibited upon deleting *URE2* in combination with *VPS34*, *VPS45*, or *PEP5* was not very surprising. The *ure2Δvps3Δ* double mutant exhibited a WT transcription profile under derepressive conditions, thus, suggesting that Vps3 may be dispensable for GATA factor function, at least when Ure2 is absent. Multiple speculative explanations are possible. Among them, Vps components could be incorporated in a membrane-based nitrogen sensing system where mutating the Vps components would impair transmission of the signal and thus leading to constitutive negative regulation of the GATA factors in a Ure2-dependent manner. However, adding a *ure2* mutation to a *vps* mutation (i.e., *ure2Δvps3Δ* double mutant) will relieve the negative regulation of the GATA factors. Accordingly, Ure2 would appear to function downstream of the VPS system. Another possibility might be that Vps proteins and Ure2 might exert GATA factor regulation through separate pathways. Taking advantage of the inability of the *ure2Δvps3Δ* double mutant to grow on YNB-ammonia medium, it could prove useful to select suppressors or mutants able to bypass this lethality. This would enable the identification of new components involved either in GATA factor regulatory pathways or in membrane trafficking as well as other potential unrelated roles of Ure2.

In *S. cerevisiae*, some, but not all, amino acids are compartmentalized in the vacuoles (Wiemken and Durr 1974; Kitamoto et al. 1988; Sekito et al. 2008). Major known

regulators of GATA factor-mediated gene activation (TORC1, Tap42-Sit4 and Gln3) have been localized to membranes of the secretory pathway (Cardenas and Heitman 1995; Kunz et al. 2000; Chen and Kaiser 2003; Wedaman et al. 2003; Reinke et al. 2004; Yan et al. 2006; Aronova et al. 2007; Puria et al. 2008). Moreover, the EGO complex, which is thought to couple amino acid signals to TORC1 activity, resides on the vacuolar membrane (Binda et al. 2009). Together, our results and these observations are consistent with the conclusion that Class C and D Vps proteins are required for the responses of GATA factor intracellular localization and function to changing nitrogen availability. Further, the data suggest that cytoplasmic membranes, which are associated with Golgi-to-vacuolar trafficking, appear to be additionally involved with the generation and/or implementation of these responses.

Together with our data, a recent report (Han and Emr 2011) supports the growing view that transcription factor regulation occurs not only inside the nucleus, but potentially at cytoplasmic compartments as well. In this manner, cytoplasmic signals may directly or indirectly regulate the function of transcription factors, thus strengthening the connection between gene expression and the intra- and extracellular environments to which it responds.

Acknowledgments

We thank André Feller for preparing the artwork, Anna Maria Marini for useful suggestions to improve the manuscript, and Bruno André for allowing the use of the fluorescence microscope. This work was supported by the Commission Communautaire Française (COCOF; E. D., F. V., and I. G.) and the Fonds de la Recherche Fondamentale Collective (FRFC 2.4547.11; E. D., and I. G.) and National Institute of General Medical Sciences grant GM-35642 (J. J. T. and T. G. C.). M. F. K. is an FNRS Research Fellow (Fonds de la Recherche Scientifique).

Conflict of Interest

None declared.

References

- Aronova, S., K. Wedaman, S. Anderson, J. Yates III, and T. Powers. 2007. Probing the membrane environment of the TOR kinases reveals functional interactions between TORC1, actin, and membrane trafficking in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 18:2779–2794.
- Backer, J. M. 2008. The regulation and function of Class III PI3Ks: novel roles for Vps34. *Biochem. J.* 410:1–17.

- Becherer, K. A., S. E. Rieder, S. D. Emr, and E. W. Jones. 1996. Novel syntaxin homologue, Pep12p, required for the sorting of luminal hydrolases to the lysosome-like vacuole in yeast. *Mol. Biol. Cell* 7:579–594.
- Beck, T., and M. N. Hall. 1999. The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* 402:689–692.
- Binda, M., M. P. Peli-Gulli, G. Bonfils, N. Panchaud, J. Urban, T. W. Sturgill, et al. 2009. The Vam6 GEF controls TORC1 by activating the EGO complex. *Mol. Cell* 35:563–573.
- Blinder, D., P. W. Coschigano, and B. Magasanik. 1996. Interaction of the GATA factor Gln3p with the nitrogen regulator Ure2p in *Saccharomyces cerevisiae*. *J. Bacteriol.* 178:4734–4736.
- Bonfils, G., M. Jaquenoud, S. Bontron, C. Ostrowicz, C. Ungermann, and C. De Virgilio. 2012. Leucyl-tRNA synthetase controls TORC1 via the EGO complex. *Mol. Cell* 46:105–110.
- Bowers, K., and T. H. Stevens. 2005. Protein transport from the late Golgi to the vacuole in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1744:438–454.
- Byfield, M. P., J. T. Murray, and J. M. Backer. 2005. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J. Biol. Chem.* 280:33076–33082.
- Cardenas, M. E., and J. Heitman. 1995. FKBP12-rapamycin target TOR2 is a vacuolar protein with an associated phosphatidylinositol-4 kinase activity. *EMBO J.* 14: 5892–5907.
- Cardenas, M. E., N. S. Cutler, M. C. Lorenz, C. J. Di Como, and J. Heitman. 1999. The TOR signaling cascade regulates gene expression in response to nutrients. *Genes Dev.* 13:3271–3279.
- Chen, E. J., and C. A. Kaiser. 2003. LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. *J. Cell Biol.* 161:333–347.
- Coffman, J. A., R. Rai, and T. G. Cooper. 1995. Genetic evidence for Gln3p-independent, nitrogen catabolite repression-sensitive gene expression in *Saccharomyces cerevisiae*. *J. Bacteriol.* 177:6910–6918.
- Coffman, J. A., R. Rai, T. Cunningham, V. Svetlov, and T. G. Cooper. 1996. Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 16:847–858.
- Cooper, T. G. 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*. Pp. 39–99 in J. N. Strathern, E. W. Jones and J. R. Broach, eds. *The molecular biology of the yeast saccharomyces: metabolism and gene expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Cooper, T. G. 2002. Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. *FEMS Microbiol. Rev.* 26:223–238.
- Costanzo, M., A. Baryshnikova, J. Bellay, Y. Kim, E. D. Spear, C. S. Sevier, et al. 2010. The genetic landscape of a cell. *Science* 327:425–431.
- Courchesne, W. E., and B. Magasanik. 1988. Regulation of nitrogen assimilation in *Saccharomyces cerevisiae*: roles of the URE2 and GLN3 genes. *J. Bacteriol.* 170:708–713.
- Cox, K. H., R. Rai, M. Distler, J. R. Daugherty, J. A. Coffman, and T. G. Cooper. 2000. *Saccharomyces cerevisiae* GATA sequences function as TATA elements during nitrogen catabolite repression and when Gln3p is excluded from the nucleus by overproduction of Ure2p. *J. Biol. Chem.* 275:17611–17618.
- Cox, K. H., J. J. Tate, and T. G. Cooper. 2002. Cytoplasmic compartmentation of Gln3 during nitrogen catabolite repression and the mechanism of its nuclear localization during carbon starvation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 277:37559–37566.
- Cox, K. H., J. J. Tate, and T. G. Cooper. 2004. Actin cytoskeleton is required for nuclear accumulation of Gln3 in response to nitrogen limitation but not rapamycin treatment in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 279:19294–19301.
- Cunningham, T. S., R. Andhare, and T. G. Cooper. 2000. Nitrogen catabolite repression of DAL80 expression depends on the relative levels of Gat1p and Ure2p production in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 275:14408–14414.
- Devasahayam, G., D. Ritz, S. B. Helliwell, D. J. Burke, and T. W. Sturgill. 2006. Pmr1, a Golgi Ca²⁺/Mn²⁺-ATPase, is a regulator of the target of rapamycin (TOR) signaling pathway in yeast. *Proc. Natl. Acad. Sci. USA* 103:17840–17845.
- Drillien, R., and F. Lacroute. 1972. Ureidosuccinic acid uptake in yeast and some aspects of its regulation. *J. Bacteriol.* 109:203–208.
- Dubouloz, F., O. Deloche, V. Wanke, E. Cameroni, and C. De Virgilio. 2005. The TOR and EGO Protein Complexes Orchestrate Microautophagy in Yeast. *Mol. Cell* 19:15–26.
- Feller, A., M. Boeckstaens, A. M. Marini, and E. Dubois. 2006. Transduction of the nitrogen signal activating Gln3-mediated transcription is independent of Npr1 kinase and Rsp5-Bul1/2 ubiquitin ligase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 281:28546–28554.
- Feller, A., I. Georis, J. J. Tate, T. G. Cooper, and E. Dubois. 2013. Alterations in the Ure2 alphaCap domain elicit different GATA factor responses to rapamycin treatment and nitrogen limitation. *J. Biol. Chem.* 288:1841–1855.
- Gao, M., and C. A. Kaiser. 2006. A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. *Nat. Cell Biol.* 8:657–667.
- Georis, I., J. J. Tate, T. G. Cooper, and E. Dubois. 2008. Tor pathway control of the nitrogen-responsive DAL5 gene bifurcates at the level of Gln3 and Gat1 regulation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 283:8919–8929.

- Georis, I., A. Feller, F. Vierendeels, and E. Dubois. 2009. The yeast GATA factor Gat1 occupies a central position in nitrogen catabolite repression-sensitive gene activation. *Mol. Cell. Biol.* 29:3803–3815.
- Georis, I., J. J. Tate, T. G. Cooper, and E. Dubois. 2011a. Nitrogen-responsive regulation of GATA protein family activators Gln3 and Gat1 occurs by two distinct pathways, one inhibited by rapamycin and the other by methionine sulfoximine. *J. Biol. Chem.* 286:44897–44912.
- Georis, I., J. J. Tate, A. Feller, T. G. Cooper, and E. Dubois. 2011b. Intranuclear function for protein phosphatase 2A: Pph21 and Pph22 are required for rapamycin-induced GATA factor binding to the DAL5 promoter in yeast. *Mol. Cell. Biol.* 31:92–104.
- Grenson, M., E. Dubois, M. Piotrowska, R. Drillien, and M. Aigle. 1974. Ammonia assimilation in *Saccharomyces cerevisiae* as mediated by the two glutamate dehydrogenases. Evidence for the *gdhA* locus being a structural gene for the NADP-dependent glutamate dehydrogenase. *Mol. Gen. Genet.* 128:73–85.
- Han, B. K., and S. D. Emr. 2011. Phosphoinositide [PI(3,5)P₂] lipid-dependent regulation of the general transcriptional regulator Tup1. *Genes Dev.* 25:984–995.
- Hardwick, J. S., F. G. Kuruvilla, J. K. Tong, A. F. Shamji, and S. L. Schreiber. 1999. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc. Natl. Acad. Sci. USA* 96:14866–14870.
- Hoppins, S., S. R. Collins, A. Cassidy-Stone, E. Hummel, R. M. Devay, L. L. Lackner, et al. 2011. A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J. Cell Biol.* 195:323–340.
- Kihara, A., T. Noda, N. Ishihara, and Y. Ohsumi. 2001. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in *Saccharomyces cerevisiae*. *J. Cell Biol.* 152:519–530.
- Kim, E., P. Goraksha-Hicks, L. Li, T. P. Neufeld, and K. L. Guan. 2008. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat. Cell Biol.* 10:935–945.
- Kitamoto, K., K. Yoshizawa, Y. Ohsumi, and Y. Anraku. 1988. Dynamic aspects of vacuolar and cytosolic amino acid pools of *Saccharomyces cerevisiae*. *J. Bacteriol.* 170:2683–2686.
- Kunz, J., U. Schneider, I. Howald, A. Schmidt, and M. N. Hall. 2000. HEAT repeats mediate plasma membrane localization of Tor2p in yeast. *J. Biol. Chem.* 275:37011–37020.
- Ljungdahl, P. O., and B. Daignan-Fornier. 2012. Regulation of amino acid, nucleotide, and phosphate metabolism in *Saccharomyces cerevisiae*. *Genetics* 190:885–929.
- Magasanik, B., and C. A. Kaiser. 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* 290:1–18.
- Mitchell, A. P., and B. Magasanik. 1984. Regulation of glutamine-repressible gene products by the GLN3 function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4:2758–2766.
- Nobukuni, T., M. Joaquin, M. Rocco, S. G. Dann, S. Y. Kim, P. Gulati, et al. 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc. Natl. Acad. Sci. USA* 102:14238–14243.
- Peterson, M. R., and S. D. Emr. 2001. The class C Vps complex functions at multiple stages of the vacuolar transport pathway. *Traffic* 2:476–486.
- Prescianotto-Baschong, C., and H. Riezman. 2002. Ordering of compartments in the yeast endocytic pathway. *Traffic* 3:37–49.
- Pringle, J. R., A. E. Adams, D. G. Drubin, and B. K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565–602.
- Puria, R., and M. E. Cardenas. 2008. Rapamycin bypasses vesicle-mediated signaling events to activate Gln3 in *Saccharomyces cerevisiae*. *Commun. Integr. Biol.* 1:23–25.
- Puria, R., S. A. Zurita-Martinez, and M. E. Cardenas. 2008. Nuclear translocation of Gln3 in response to nutrient signals requires Golgi-to-endosome trafficking in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 105:7194–7199.
- Rai, R., J. J. Tate, I. Georis, E. Dubois, and T. G. Cooper. 2013a. Constitutive and Nitrogen Catabolite Repression-Sensitive Production of Gat1 Isoforms. *J. Biol. Chem.* 289:2918–2933.
- Rai, R., J. J. Tate, D. R. Nelson, and T. G. Cooper. 2013b. *gln3* mutations dissociate responses to nitrogen limitation (nitrogen catabolite repression) and rapamycin inhibition of TorC1. *J. Biol. Chem.* 288:2789–2804.
- Reinke, A., S. Anderson, J. M. McCaffery, J. Yates III, S. Aronova, S. Chu, et al. 2004. TOR complex 1 includes a novel component, Tco89p (YPL180w), and cooperates with Ssd1p to maintain cellular integrity in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 279:14752–14762.
- Rieder, S. E., and S. D. Emr. 1997. A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. *Mol. Biol. Cell* 8:2307–2327.
- Sekito, T., Y. Fujiki, Y. Ohsumi, and Y. Kakinuma. 2008. Novel families of vacuolar amino acid transporters. *IUBMB Life* 60:519–525.
- Srivastava, A., C. A. Woolford, and E. W. Jones. 2000. Pep3p/Pep5p complex: a putative docking factor at multiple steps of vesicular transport to the vacuole of *Saccharomyces cerevisiae*. *Genetics* 156:105–122.
- Stanbrough, M., D. W. Rowen, and B. Magasanik. 1995. Role of the GATA factors Gln3p and Nil1p of *Saccharomyces cerevisiae* in the expression of nitrogen-regulated genes. *Proc. Natl. Acad. Sci. USA* 92:9450–9454.
- Tate, J. J., and T. G. Cooper. 2007. Stress-responsive Gln3 localization in *Saccharomyces cerevisiae* is separable from and can overwhelm nitrogen source regulation. *J. Biol. Chem.* 282:18467–18480.
- Tate, J. J., and T. G. Cooper. 2008. Formalin can alter the intracellular localization of some transcription factors in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 8:1223–1235.

- Tate, J. J., and T. G. Cooper. 2013. Five Conditions Commonly Used to Down-regulate Tor Complex 1 Generate Different Physiological Situations Exhibiting Distinct Requirements and Outcomes. *J. Biol. Chem.* 288:27243–27262.
- Tate, J. J., R. Rai, and T. G. Cooper. 2005. Methionine sulfoximine treatment and carbon starvation elicit Snf1-independent phosphorylation of the transcription activator Gln3 in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280:27195–27204.
- Tate, J. J., A. Feller, E. Dubois, and T. G. Cooper. 2006. *Saccharomyces cerevisiae* Sit4 phosphatase is active irrespective of the nitrogen source provided, and Gln3 phosphorylation levels become nitrogen source-responsive in a sit4-deleted strain. *J. Biol. Chem.* 281:37980–37992.
- Tate, J. J., I. Georis, A. Feller, E. Dubois, and T. G. Cooper. 2009. Rapamycin-induced Gln3 dephosphorylation is insufficient for nuclear localization: Sit4 and PP2A phosphatases are regulated and function differently. *J. Biol. Chem.* 284:2522–2534.
- Tate, J. J., I. Georis, E. Dubois, and T. G. Cooper. 2010. Distinct Phosphatase Requirements and GATA Factor Responses to Nitrogen Catabolite Repression and Rapamycin Treatment in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 285:17880–17895.
- Urban, J., A. Soulard, A. Huber, S. Lippman, D. Mukhopadhyay, O. Deloche, et al. 2007. Sch9 is a major target of TORC1 in *Saccharomyces cerevisiae*. *Mol. Cell* 26:663–674.
- Wach, A. 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. *Yeast* 12:259–265.
- Wedaman, K. P., A. Reinke, S. Anderson, J. Yates III, J. M. McCaffery, and T. Powers. 2003. Tor kinases are in distinct membrane-associated protein complexes in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 14:1204–1220.
- Wiame, J. M., M. Grenson, and H. N. Jr Arst. 1985. Nitrogen catabolite repression in yeasts and filamentous fungi. *Adv. Microb. Physiol.* 26:1–88.
- Wiemken, A., and M. Durr. 1974. Characterization of amino acid pools in the vacuolar compartment of *Saccharomyces cerevisiae*. *Arch. Microbiol.* 101:45–57.
- Yan, G., X. Shen, and Y. Jiang. 2006. Rapamycin activates Tap42-associated phosphatases by abrogating their association with Tor complex 1. *EMBO J.* 25: 3546–3555.
- Zurita-Martinez, S. A., R. Puria, X. Pan, J. D. Boeke, and M. E. Cardenas. 2007. Efficient Tor signaling requires a functional class C Vps protein complex in *Saccharomyces cerevisiae*. *Genetics* 176:2139–2150.