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LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway

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LST8, a *Saccharomyces cerevisiae* gene encoding a 34-kD WD-repeat protein, was identified by mutations that caused defects in sorting Gap1p to the plasma membrane. Here, we report that the Gap1p sorting defect in the *lst8-1* mutant results from derepression of Rtg1/3p activity and the subsequent accumulation of high levels of intracellular amino acids, which signal Gap1p sorting to the vacuole. To identify the essential function of Lst8p, we isolated *lst8* mutants that are temperature-sensitive for growth. These mutants show hypersensitivity to rapamycin and derepressed Gln3p activity like cells with compromised TOR pathway activity. Like *tor2* mutants, *lst8* mutants also have cell wall integrity defects. Confirming a role for Lst8p in the TOR pathway, we find that Lst8p associates with both Tor1p and Tor2p and is a peripheral membrane protein that localizes to endosomal or Golgi membranes and cofractionates with Tor1p. Further, we show that a sublethal concentration of rapamycin mimics the Gap1p sorting defect of an *lst8* mutant. Finally, the different effects of *lst8* alleles on the activation of either the Rtg1/3p or Gln3p transcription factors reveal that these two pathways constitute distinct, genetically separable outputs of the Tor–Lst8 regulatory complex.

Introduction

The general amino acid permease Gap1p of *Saccharomyces cerevisiae* is a high-capacity permease that can transport all amino acids (Grenson et al., 1970; Jauniaux and Grenson, 1990). The nitrogen source in the growth medium regulates Gap1p activity both transcriptionally and post-transcriptionally (Magasanik and Kaiser, 2002). Growth on rich nitrogen sources such as glutamine or yeast extract/peptone/dextrose (YPD)* represses *GAP1* transcription, whereas nitrogen starvation or growth on poor nitrogen sources induces *GAP1* transcription.

The nitrogen source in the growth medium also regulates the intracellular sorting of Gap1p. When cells are grown on glutamate or when Gap1p is artificially expressed during growth on glutamine, Gap1p is sorted to the vacuole and Gap1p activity at the plasma membrane is very low. Conversely, during growth on the nitrogen sources urea, proline, or ammonia (in the S288C background), Gap1p is sorted to the plasma membrane and Gap1p activity at the plasma membrane is high (Stanbrough and Magasanik, 1995; Roberg et al., 1997b; Chen and Kaiser, 2002). Gap1p sorting is thought to be largely regulated at the endosome or trans-Golgi stages of the secretory and endosomal trafficking pathways (Roberg et al., 1997b; Helliwell et al., 2001).

Mutations that alter Gap1p sorting can be broadly divided into two classes: mutations that affect Gap1p trafficking, and mutations that affect the production of the sorting signal (Magasanik and Kaiser, 2002). The first class of mutations resides mainly in genes involved in ubiquitination, such as *BUL1*, *BUL2*, *RSP5*, and *DOA4*. Apparently, polyubiquitination of Gap1p is required for its sorting to the vacuole. Thus, mutations that interfere with the polyubiquitination of Gap1p cause high Gap1p activity and increased sorting of Gap1p to the plasma membrane (Helliwell et al., 2001; Soetens et al., 2001; Springael et al., 2002).

The second class of mutations that affect Gap1p sorting resides in genes that influence the net amount of amino acid biosynthesis, such as *GDH1*, *GLN1*, and *MKS1* (Chen and Kaiser, 2002). Yeast uses glutamate and glutamine as the nitrogen donors to synthesize all other amino acids (Magasanik, 1992). Thus, mutations that affect the rate of glutamate and glutamine synthesis also affect the net synthesis of all amino acids, and therefore affect Gap1p sorting. *GDH1* encodes the anabolic glutamate dehydrogenase, the primary enzyme responsible for glutamate synthesis during growth on ammonia medium (Grenson et al., 1974). *GLN1* encodes glutamine synthetase, an essential gene for growth on medium lacking glutamine (Mitchell, 1985). Deletion of *GDH1* or mutation

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*Abbreviation used in this paper: YPD, yeast extract/peptone/dextrose.

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of *GLN1* causes a decrease in cellular glutamate and/or glutamine content and an increase in sorting of Gap1p to the plasma membrane relative to wild type. Mutation of *MKS1* has the opposite effect. *MKS1* encodes a negative regulator of the Rtg1/3p transcription factors that control the expression of the TCA cycle enzymes responsible for α-ketoglutarate synthesis during growth on medium with glucose (Liu and Butow, 1999; Dilova et al., 2002; Sekito et al., 2002; Tate et al., 2002). Because α-ketoglutarate forms the carbon skeleton from which glutamate and glutamine are derived, *MKS1* has a net negative effect on glutamate and glutamine synthesis. Thus, an *mks1*Δ mutant shows high intracellular amino acid levels and decreased sorting of Gap1p to the plasma membrane (Chen and Kaiser, 2002).

One of the first mutants with a defect in Gap1p trafficking that we isolated was the *lst8-1* mutant. The *lst8-1* mu-

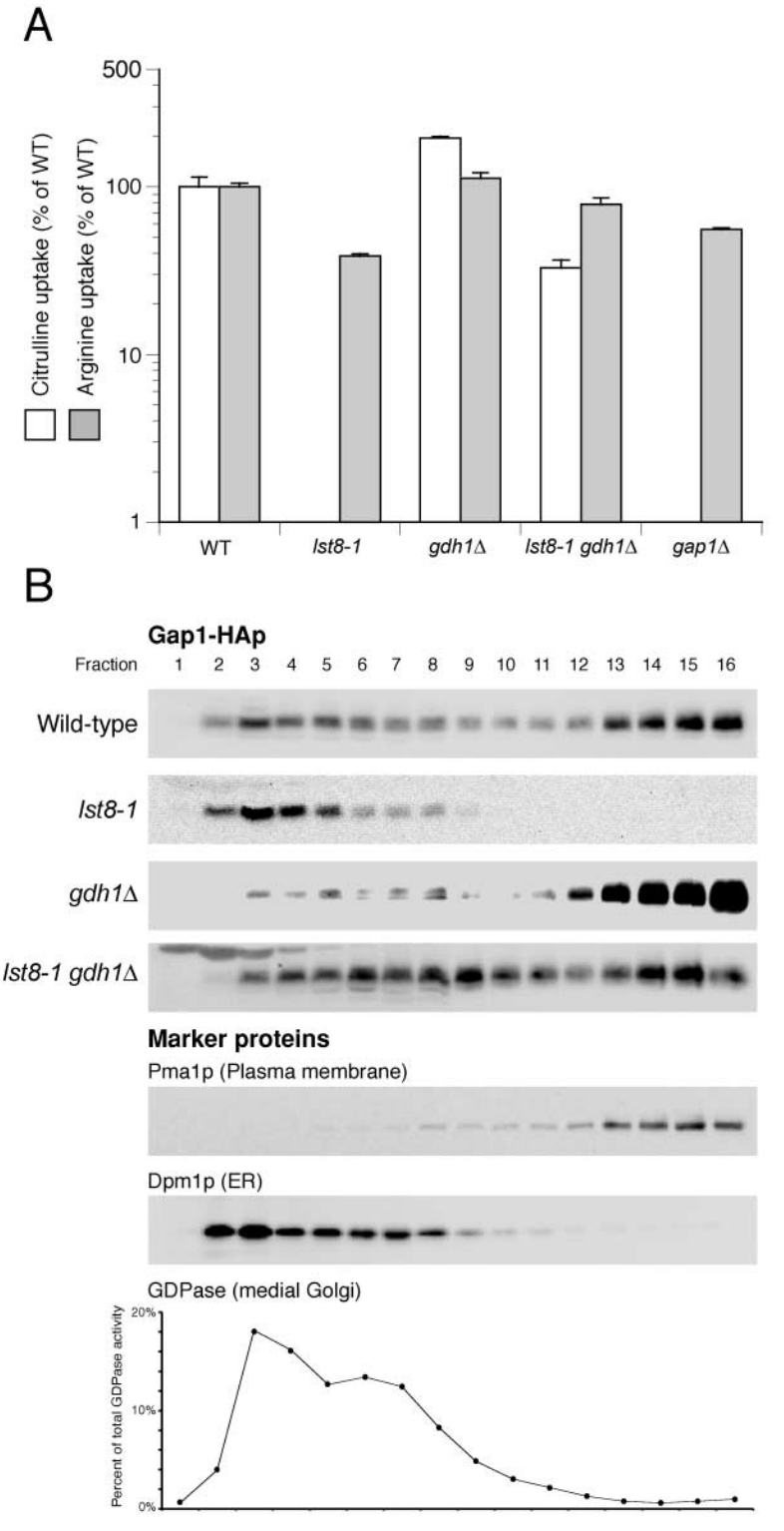

Figure 1. **The Gap1p sorting defect of an *lst8-1* mutant is suppressed by deletion of *gdh1*.** (A) Gap1p activity was measured by assaying the rate of [^{14}C]citrulline uptake (white bars) of wild-type (CKY759), *lst8-1* (CKY768), *gdh1*Δ (CKY762), or *lst8-1 gdh1*Δ (CKY769), all with the *GAP1* locus replaced by P_{ADH1}-GAP1-HA, or of *gap1*Δ (CKY445) growing on ammonia medium. The rate of [^{14}C]arginine uptake (gray bars) is shown for comparison. Uptake rates are expressed as a percentage of the uptake rate of wild-type. The data shown represent three independent assays, and the error bars represent one SD. (B) The first four strains shown in A were grown in ammonia medium at 24°C, and cell extracts were subjected to isopycnic fractionation on continuous 20–60% sucrose density gradients with EDTA. Fractions were collected from the top of the gradients and proteins were separated by SDS-PAGE. In all the strains shown, Pma1p, Dpm1p, and GDPase fractionated similarly.

tant was shown to greatly diminish sorting of Gap1p to the plasma membrane in cells grown on ammonia or urea as a nitrogen source (Roberg et al., 1997a). *LST8* encodes an essential protein with WD-repeats and has a closely related human orthologue. A recent report from the Butow lab showed that Lst8p was involved in the negative regulation of the Rtg1/3p transcription factors (Liu et al., 2001). The *lst8* mutants they isolated were shown to have elevated *CIT2* transcription and decreased sensitivity to glutamate for *CIT2* repression (Liu et al., 2001). These findings suggested that either Lst8p has an indirect effect on Gap1p sorting, like Mks1p, or that Lst8p has at least two separate functions: one function in the regulation of Rtg1/3p, and another function in the regulation of permease sorting.

Recently, Hall and colleagues reported that Lst8p was associated with Tor1p and with Tor2p. Tor1p coimmunoprecipitated with Kog1p and Lst8p, and Tor2p coimmunoprecipitated with Kog1p, Lst8p, Avo1p, Avo2p, and Avo3p. These coimmunoprecipitation studies, supplemented by depletion assays with genes under the control of glucose-repressible promoters, led Hall and colleagues to propose that Lst8p associates with the Tor proteins in the TORC1 and TORC2 complexes, which may have distinct roles in growth control (Loewith et al., 2002).

Here, we investigate the role of Lst8p in Gap1p permease sorting and find that the effects of *lst8* mutations on Gap1p sorting are an indirect consequence of increased intracellular amino acid levels in *lst8* mutants. We characterize the phenotypes of *lst8* mutants and present evidence that Lst8p is a positively acting component of Tor-containing complexes.

Results

The effect of *lst8-1* on Gap1p sorting is indirect

An *lst8-1* mutation causes Gap1p to be sorted to the vacuole under growth conditions in which Gap1p is normally sorted to the plasma membrane (Roberg et al., 1997a). Because Gap1p is both transcriptionally and post-transcriptionally regulated by the nitrogen source in the growth medium, we verified that the primary effect of *lst8-1* on Gap1p activity was an effect on Gap1p sorting, using a P_{ADH1}-HA-GAP1 construct that renders *GAP1* transcription insensitive to nitrogen source quality (Chen and Kaiser, 2002). Indeed, an *lst8-1* mutant with P_{ADH1}-HA-GAP1 had no detectable Gap1p activity as measured by uptake of [^{14}C]citrulline, which is transported exclusively by Gap1p (Fig. 1 A), and no Gap1p localized to the plasma membrane (Fig. 1 B). Uptake of [^{14}C]arginine, which is transported by Gap1p and by the arginine permease Can1p, is similar in the *lst8-1* and *gap1*Δ mutants, indicating that the effect of *lst8-1* on permease sorting is specific for Gap1p, and is not the result of a general decrease in all permease activity (Fig. 1 A).

Butow and colleagues recently reported that Lst8p negatively regulates the Rtg1p and Rtg3p transcription factors, which control the transcription of genes responsible for α-ketoglutarate synthesis during growth on medium with glucose (Liu et al., 2001). Glutamate and glutamine normally repress Rtg1/3p activity in a negative feedback loop required for proper amino acid homeostasis because α-ketoglutarate is the precursor of glutamate and glutamine, the two amino acids from which the cell synthesizes all the other amino acids (Magasanik, 1992; Liu and Butow, 1999; Komeili et al., 2000). Deletion of *MKS1*, another negative regulator of Rtg1/3p, has been shown to result in elevated intracellular levels of α-ketoglutarate, glutamate, and total amino acids, and as a consequence leads to decreased sorting of Gap1p to the plasma membrane (Feller et al., 1997; Chen and Kaiser, 2002). To determine if the *lst8-1* mutation had an effect similar to *mks1*Δ on cellular amino acid content, we performed whole-cell amino acid analysis and found that *lst8-1*, like *mks1*Δ, had ~1.5–2.5-fold higher intracellular levels of glutamate and total amino acids than wild-type cells (Table I).

If an *lst8-1* mutation causes a Gap1p sorting defect indirectly by de-repressing Rtg1/3p and increasing cellular glutamate levels, then the elimination of *GDH1,* which encodes the major enzyme for the synthesis of glutamate from α-ketoglutarate on ammonia medium (Grenson et al., 1974), should significantly diminish the Gap1p sorting defect of an *lst8-1* mutant. We found that the *lst8-1 gdh1*Δ double mutant had 30% of wild-type Gap1p activity, compared with <1% for the *lst8-1* mutant alone during growth on ammonia (Fig. 1 A). The *lst8-1 gdh1*Δ double mutant also showed more Gap1p sorted to the plasma membrane than an *lst8-1* mutant (Fig. 1 B). Together with the amino acid analysis of *lst8-1*, these data suggested that the Gap1p sorting defect was an indirect consequence of the increased amino acid levels in the *lst8-1* mutant.

Identification of temperature-sensitive *lst8* mutations

LST8 is an essential gene, and we generated conditional alleles to investigate the essential function of *LST8*. The *lst8-6*

Table I. **Total amino acid content of strains relative to wild type**

	Amino acid content (normalized to WT)			
	Glt	Gln	Glt + Gln	Total amino acids
WT	1.0	1.0	1.0	1.0
lst8-1	2.4 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	1.7 ± 0.1
*mks1*Δ	2.4 ± 0.2	1.3 ± 0.1	1.8 ± 0.1	1.5 ± 0.1
WT + 5 ng/ml rapamycin	2.5 ± 0.3	4.5 ± 0.1	6.6 ± 0.0	2.6 ± 0.0

Amino acid contents of *lst8-1* and *mks1*Δ and WT + rap samples are expressed relative to a wild-type amino acid content of 1. Absolute values of WT amino acid content (no drug) are as follows (nmol/OD_{600}): Glt, 22.3 ± 4.6; Gln, 33.1 ± 6.8; Glt + Gln, 55.3 ± 10.3; and Total, 134.6 ± 26.4. This experiment was performed four times with *lst8-1* and twice with *mks1*Δ and WT + 5 ng/ml rapamycin. Wild-type (CKY443), *lst8-1* (CKY526), and *mks1*Δ (CKY758) were grown to exponential phase on minimal ammonia media at 24°C. In addition, wild-type was grown in the same medium containing 5 ng/ml rapamycin.

Table II. **Mutations in *lst8* cause derepression of Rtg1/3p-dependent transcription**

Strain	P_{CIT2}-LacZ expression on glutamate medium	Standard deviation
	(β-galactosidase units)	*(β-galactosidase units)*
WT	2.4	0.6
lst8-1	9.3	4.1
lst8-6	251.3	10.7
lst8-7	83.9	8.8
*mks1*Δ	729.1	39.7
*ure2*Δ	4.2	1.5
*rtg2*Δ	0.8	0.1
*rtg2*Δ *lst8-1*	1.4	0.8
*rtg2*Δ *lst8-6*	147.8	13.9
*rtg2*Δ *lst8-7*	33.7	2.4
*rtg2*Δ *mks1*Δ	752.4	80.4

Strains were *ura3-52* (CKY772), *lst8-1 ura3-52* (CKY773), *lst8-6 ura3-52* (CKY774), *lst8-7 ura3-52* (CKY775), *mks1*Δ *ura3-52* (CKY776), *ure2*Δ *ura3-52* (CKY777), *rtg2*Δ *ura3-52* (CKY795), *rtg2*Δ *lst8-1 ura3-52* (CKY796), *rtg2*Δ *lst8-6 ura3-52* (CKY797), *rtg2*Δ *lst8-7 ura3-52* (CKY798), and *rtg2*Δ *mks1*Δ *ura3-52* (CKY799) containing pEC261.

Table III. **Mutations in *lst8* cause increased Gln3p-dependent transcription**

Strain	P_{GAP1}-LacZ expression in glutamine medium	Standard deviation
	(β-galactosidase units)	*(β-galactosidase units)*
WT	12.7	0.4
lst8-1	35.9	5.8
lst8-6	14.6	1.5
lst8-7	31.3	2.5
*mks1*Δ	0.4	0.1
*ure2*Δ	361.4	78.9
*tor1*Δ	24.6	6.0
WT + 200 ng/ml rap (1 h)	25.1	1.5
WT + 200 ng/ml rap (2 h)	82.2	2.3

Strains were as listed in Table II and *tor1*Δ *ura3-52* (CKY794), containing pMS29.

and *lst8-7* temperature-sensitive alleles were generated by hydroxylamine and PCR mutagenesis, respectively, and integrated at the *LST8* locus. The *lst8-6* mutant failed to grow at temperatures above 34°C, whereas *lst8-7* failed to grow above 37°C on YPD medium. Like *lst8-1*, both *lst8-6* and *lst8-7* had low Gap1p activity at the plasma membrane during growth on ammonia medium at 24°C (unpublished data).

We first checked *lst8-6* and *lst8-7* for derepressed Rtg1/3p activity by using a P_{CIT2}-LacZ reporter. Rtg1/3p activity is normally repressed in the presence of glutamate, but has been reported to be derepressed in several different *lst8* mutants grown in YPD or in medium with casamino acids (Liu et al., 2001). We found that *lst8-6* and *lst8-7* had strongly derepressed P_{CIT2}-LacZ expression during growth on glutamate medium (Table II). Consistent with previous reports, we found that the *lst8-1* allele showed little Rtg1/3p derepression, and that overall the *lst8* mutants had a more modest effect on Rtg1/3p derepression on glutamate medium than *mks1*Δ (Liu et al., 2001; Sekito et al., 2002). The *lst8-6* and *lst8-7* mutants were able to suppress *rtg2*Δ somewhat with regard to P_{CIT2}-LacZ activity, though suppression was not complete as in the case of *mks1*Δ (Table II; Liu et al., 2001; Sekito et al., 2002). Also, *lst8-6* and *lst8-7* (but not *lst8-1*) complemented the glutamate auxotrophy of *rtg2*Δ (unpublished data).

We looked for a link between Lst8p function and the activity of the TOR pathway by testing the sensitivity of *lst8* mutants to rapamycin. Like Lst8p and Mks1p, the TOR pathway negatively regulates Rtg1/3p, as shown by the ability of the TOR inhibitor rapamycin to induce the expression of Rtg1/3p-dependent genes in strains grown on glutamate or glutamine (Komeili et al., 2000). At the semi-permissive temperature of 30°C on rich medium, *lst8* mutants were hypersensitive to rapamycin (Fig. 2), like a *tor1*Δ mutant that was previously shown to be rapamycin hypersensitive (Chan et al., 2000), indicating that Lst8p might function positively in the TOR pathway.

lst8 mutations derepress the Gln3p transcription factor

A well-studied effect of rapamycin in yeast is its ability to produce effects on the global pattern of gene expression sim-

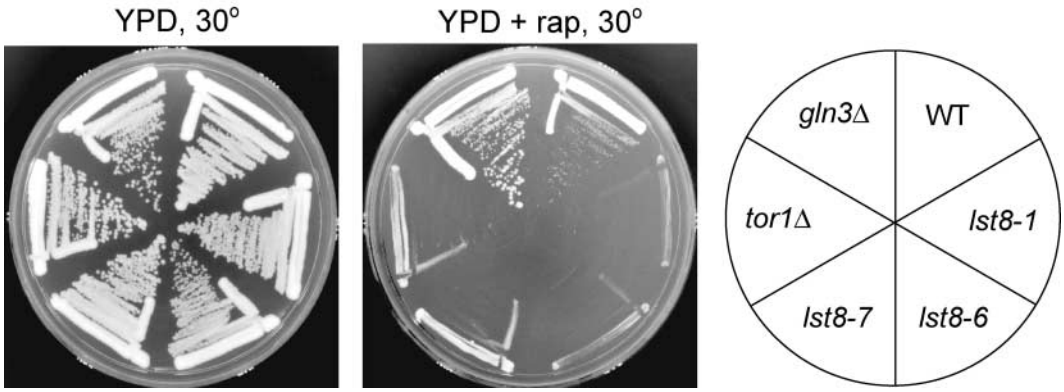

Figure 2. **Mutations in *lst8* confer rapamycin hypersensitivity.** Wild-type (CKY443), *lst8-1* (CKY526), *lst8-6* (CKY770), *lst8-7* (CKY771), *tor1*Δ (Euroscarf), and *gln3*Δ (CKY778) were streaked onto YPD or YPD + 200 ng/ml rapamycin and incubated at 30°C for 2 or 4 d, respectively. The *tor1*Δ strain is included as a control for a rapamycin-hypersensitive strain, and the *gln3*Δ strain is included as an example of a rapamycin-resistant strain.

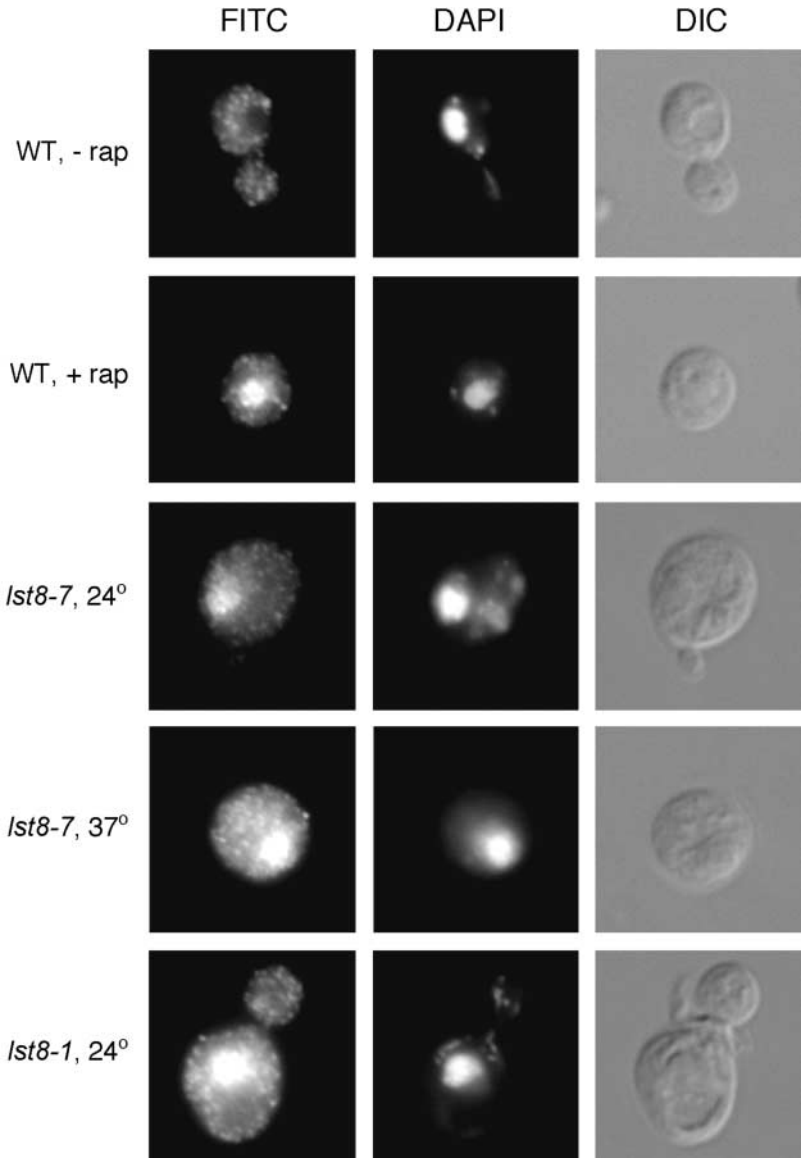

Figure 3. **Mutations in *lst8* cause nuclear localization of Gln3p during growth on glutamine.** Wild-type (CKY779), *lst8-7* (CKY781), and *lst8-1* (CKY780), all with integrated *GLN3-myc,* were grown in glutamine medium at 24°C. Rapamycin was added to the indicated sample for 30 min, and one *lst8-7* sample was incubated at 37°C for 2 h before fixation of cells for immunofluorescence. All images are shown at the same magnification.

ilar to those of nitrogen starvation (Cardenas et al., 1999; Hardwick et al., 1999). Rapamycin treatment induces the transcription of nitrogen-regulated genes such as *GAP1* that are normally repressed by good nitrogen sources. We examined P_{GAP1}-LacZ expression in *lst8* mutants grown in glutamine medium, which strongly represses *GAP1* transcription in wild-type cells. *GAP1* expression was derepressed 2.5–3-fold in *lst8-1* and *lst8-7* mutants growing on glutamine (Table III). In comparison, wild-type cells treated with rapamycin for 1 h and *tor1*Δ showed a twofold increase in P_{GAP1}-LacZ activity, and wild-type cells treated with rapamycin for 2 h showed a 6.5-fold increase in P_{GAP1}-LacZ activity. In a *ure2*Δ mutant in which the negative regulation of the Gln3p transcription factor by glutamine has been completely abolished, *GAP1* expression was derepressed ~30-fold (Table III). Unlike the *mks1*Δ and *ure2*Δ mutations, which exclusively derepress either Rtg1/3p activity or Gln3p activity, respectively, *lst8* mutants derepress both Rtg1/3p and Gln3p regulation (Table II and Table III). This result further suggested that *LST8* might function in the TOR pathway because the TOR pathway influences both Rtg1/3p and Gln3p regulation.

Derepression of *GAP1* transcription by rapamycin treatment has been shown to be accompanied by relocalization of the GATA-type transcription factor Gln3p from the cytoplasm to the nucleus (Beck and Hall, 1999). By immunofluorescence we found that, like rapamycin treatment of wild-type cells, mutation of *lst8* caused the inappropriate nuclear localization of Gln3p-myc in many cells growing in glutamine medium (Fig. 3). In our strain background growing on glutamine, we found that rapamycin treatment for 30 min caused nuclear localization in 28% of the cells, whereas the *lst8-7* and *lst8-1* mutations resulted in the nuclear localization of Gln3p in 8–19% of the cells (Table IV). Deletion of *gln3* has been shown to confer rapamycin resistance (Chan et al., 2000). We found that deletion of *gln3* could partially rescue the temperature-sensitive growth defect of *lst8-7* (Fig. 4). Together, these data show that mutation of *lst8* produces effects similar to those observed on rapamycin treatment.

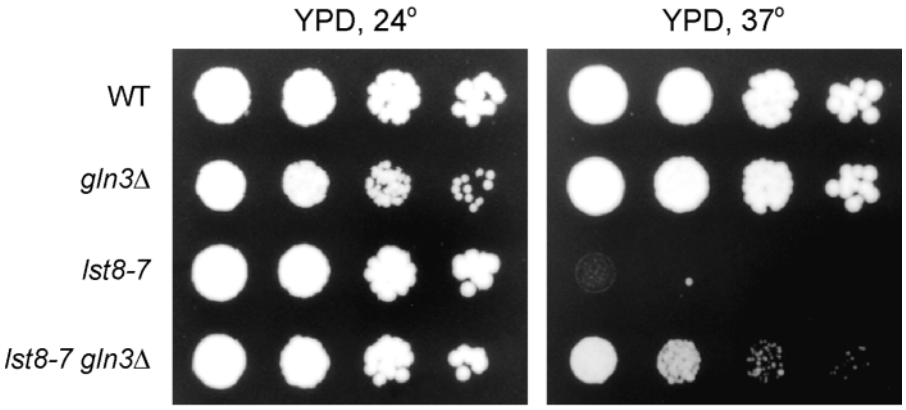

Figure 4. **Deletion of *gln3* suppresses the temperature-sensitive growth defect of *lst8-7*.** Wild-type (CKY443), *gln3*Δ (CKY778), *lst8-7* (CKY771), and *lst8-7 gln3*Δ (CKY782) were spotted onto YPD plates and grown at 24°C or 37°C for 3 d.

Cell wall defects of *lst8* mutants

Tor2p has a unique function, not shared with Tor1p, in the maintenance of the cell wall and actin cytoskeleton (Schmidt et al., 1996; Bickle et al., 1998; Helliwell et al., 1998). A *tor2* temperature-sensitive mutation is suppressed by growth of cells on media with osmotic support or with a low concentration of detergent, or by mutations in genes that are important for cell wall integrity such as *FKS1* and *CWH41,* which encode glucan synthase and glucosidase I, respectively (Bickle et al., 1998). A possible explanation for this type of suppression of *tor2* alleles is that agents or mutations that perturb the cell wall may activate a *TOR2*-independent pathway for activating Rho1p, leading to a net stabilization of the cell wall (Bickle et al., 1998).

We found that inclusion of sorbitol in the growth medium fully rescued the temperature sensitivity of *lst8-6* and *lst8-7*, indicating that these mutations were lethal at high temperature due to cell lysis (Fig. 5 A). However, inclusion of sorbitol in the growth medium did not restore growth to an *lst8*Δ mutant (unpublished data), indicating that the *lst8*Δ mutant fails to grow for reasons other than cell wall instability. We found that SDS in the growth medium or *fks1*Δ or *cwh41*Δ mutations could partially suppress the temperature sensitivity of *lst8-6* (Fig. 5, B and C). Together, these data suggest that Lst8p may also participate with Tor2p in maintaining cell wall integrity.

Lst8p associates with Tor1p and Tor2p

We tested whether Lst8p associates with Tor1p or Tor2p, given the evidence presented thus far that mutation of *lst8* causes effects similar to the mutation of *tor2* and to the inactivation of TOR pathway function with rapamycin. In mammalian cells, mLst8 was found to be associated with mTOR (Kim, D.H., and D.M. Sabatini, personal communication). HA-tagged versions of Tor1p and Tor2p were reported to be functional (Fiorentino and Crabtree, 1997; Kunz et al., 2000), and we verified the function of our constructs by complementation of the rapamycin sensitivity of a *tor1*Δ strain (for *HA-TOR1*) and complementation of the lethality of a *tor2*Δ strain (for *HA-TOR2*; unpublished data). We expressed *HA-TOR1* or *HA-TOR2* from their own promoters on *CEN* vectors in strains with *LST8-6xmyc* integrated at the *LST8* locus. Using myc antibody to precipitate Lst8p-myc–containing complexes and immunoblots to detect HA-Tor1p or HA-Tor2p, we found that HA-Tor1p and HA-Tor2p are present in complex with Lst8p-myc (Fig. 6). We also looked at the dependence of this interaction on the nitrogen source, but found that the association of HA-Tor1p and HA-Tor2p with Lst8p-myc is not significantly influenced by growth in nitrogen-free medium or in YPD with 0.3% glutamine (unpublished data). Together with the *lst8* mutant phenotypes, this result indicates that Lst8p physically associates with Tor1p and Tor2p to effect TOR pathway function.

Table IV. **Quantitation of *lst8* or rapamycin-treated cells with Gln3p localized to the nucleus, shown in Fig. 3**

Strain	Percentage of cells with nuclear Gln3p
WT, −rap	0% (n = 625)
WT, +rap	28% (n = 621)
lst8-7, 24°C	8% (n = 626)
lst8-7, 37°C	11% (n = 657)
lst8-1, 24°C	19% (n = 626)

Partial inactivation of TOR function results in low Gap1p activity

The data presented thus far lead to the prediction that a partial inactivation of the TOR pathway, like mutation of *lst8*, should lead to an increase in cellular amino acid pools and a decrease in the amount of Gap1p sorted to the plasma membrane. However, we showed recently that inhibition of Tor by the lethal concentration of rapamycin that is routinely used to arrest cell growth, halt translation initiation, and induce rapamycin-sensitive genes fails to signal nitrogen starvation to the Gap1p sorting machinery; the addition of 200 ng/ml rapamycin to rich medium failed to increase Gap1p sorting to the plasma membrane in cells with P_{ADH1}-HA-GAP1 (Chen and Kaiser, 2002). It is likely that 200 ng/ml rapamycin kills cells before cellular amino acid pools have the opportunity to increase, and a sublethal level of rapamycin might better mimic the effect of an *lst8* mutation, allowing amino acid levels to rise, and thus producing a Gap1p sorting defect.

To test this prediction, we measured Gap1p activity in a strain containing P_{ADH1}-HA-GAP1 grown for 18 h in ammonia medium with a sublethal concentration of 5 ng/ml rapamycin. At this low rapamycin concentration, the doubling time is ~90% of the doubling time seen in am-

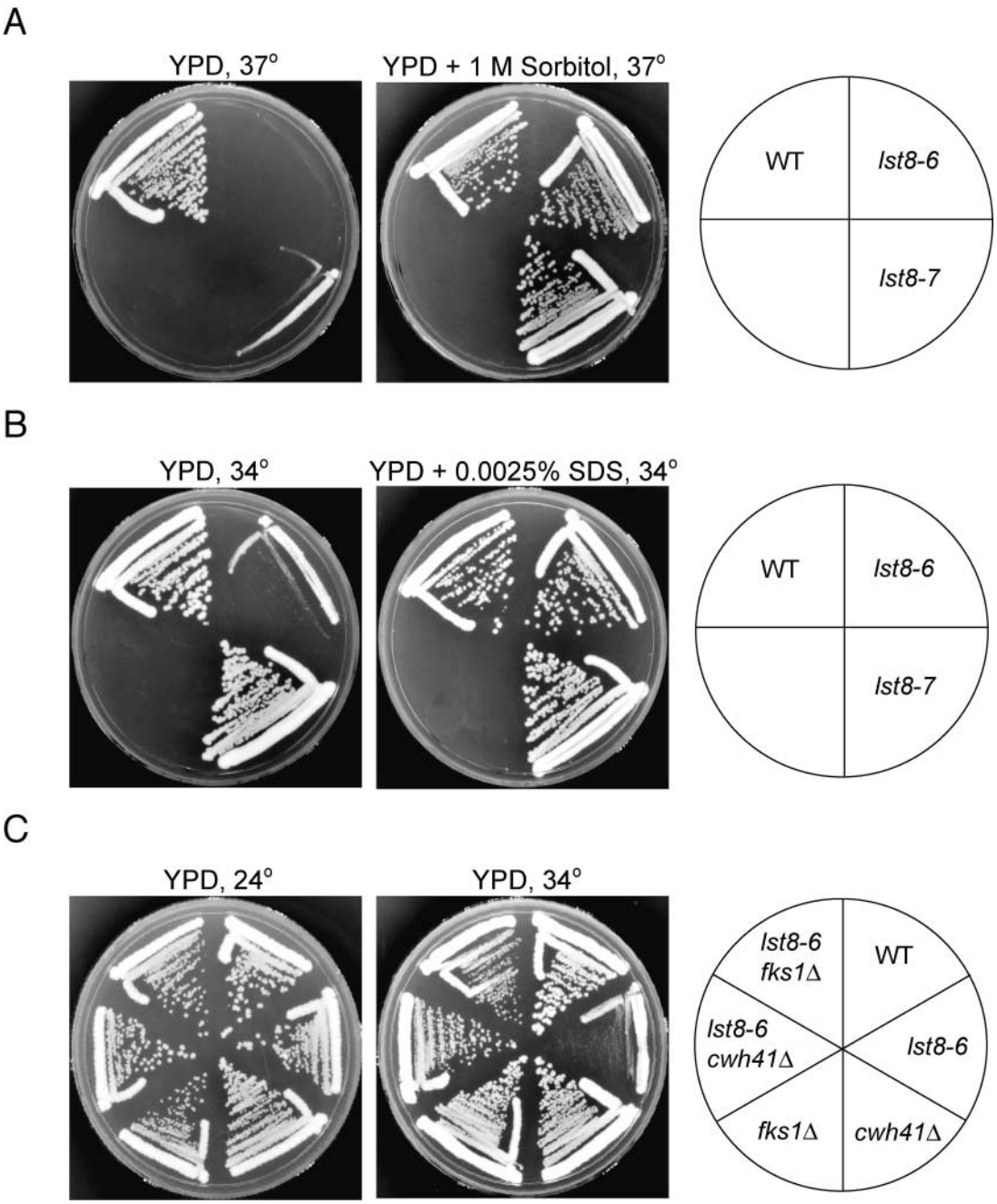

Figure 5. **Inclusion of sorbitol or SDS in the growth medium or deletion of *cwh41* or *fks1* suppresses the temperature-sensitive growth defect of *lst8* mutants.** (A) Wild-type (CKY443), *lst8-6* (CKY770), and *lst8-7* (CKY771) were streaked onto YPD or YPD + 1 M sorbitol and incubated at 37°C for 2 or 3 d, respectively. (B) The same strains as in A were streaked onto YPD or YPD + 0.0025% SDS and incubated at 34°C for 2 d. (C) Wild-type, *lst8-6*, *cwh41*Δ (Euroscarf), *fks1*Δ (Euroscarf), *lst8-6 cwh41*Δ (CKY786), *and lst8-6 fks1*Δ (CKY787) were streaked onto YPD and incubated at 24°C or 34°C for 3 or 2 d, respectively.

monia medium without rapamycin (unpublished data). Cells grown in the sublethal concentration of rapamycin showed 6% of the Gap1p activity of cells without rapamycin (Fig. 7 A), and a corresponding decrease in the amount of Gap1p localized to the plasma membrane (Fig. 7 B). As with *lst8-1*, the Gap1p sorting defect caused by the low level of rapamycin could be partially suppressed by *gdh1*Δ (Fig. 7 A). Furthermore, cells grown in ammonia medium plus 5 ng/ml rapamycin for 18 h showed a 2.6-fold increase in total amino acid content relative to cells grown without rapamycin (Table I). Thus, like mutation of *lst8*, impairment of the TOR pathway by growth with a sublethal rapamycin concentration causes increased amino acid levels and decreased Gap1p sorting to the plasma membrane.

Genetically separable effects of *lst8* mutants with the Rtg1/3 and Gln3 pathways

An examination of the *CIT2* and *GAP1* reporter assays with *lst8-1* and *lst8-6* indicated that *lst8-6* had a very strong defect

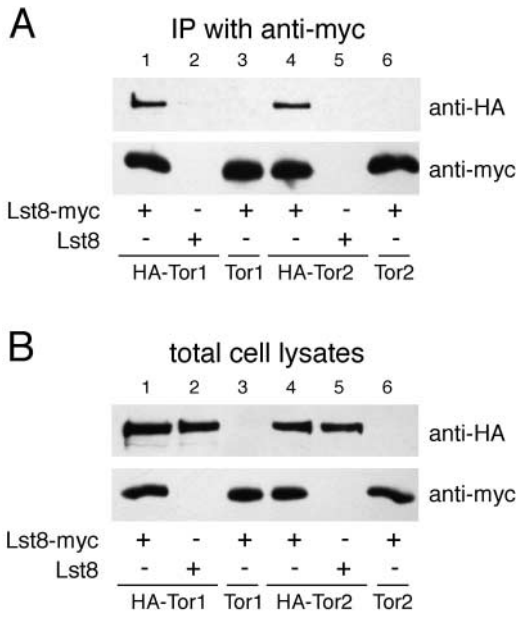

Figure 6. **Tor1p and Tor2p associate with Lst8p.** A wild-type strain with integrated *LST8-myc* from its own promoter (CKY783; lanes 1, 3, 4, and 6) or untagged *LST8* (CKY8; lanes 2 and 5) was transformed with pRS316 containing *HA-TOR1* (pEC267; lanes 1 and 2), *TOR1* (pEC262; lane 3), *HA-TOR2* (pEC268; lanes 4 and 5), or *TOR2* (pEC263; lane 6). Strains were grown in SMM-uracil at 30°C. Anti-myc (rabbit 9E10) immunoprecipitates (A) or cell lysates (B) were subjected to SDS-PAGE and Western blotting with either HA (12CA5) or myc (monoclonal 9E10) antibody.

Table V. ***P_{CIT2}-LacZ* and *P_{GAP1}-LacZ* reporter assays with additional *lst8* mutants**

Strain	P_{CIT2}-LacZ expression in glutamate medium	P_{GAP1}-LacZ expression in glutamate medium
	(β-galactosidase units)	*(β-galactosidase units)*
WT	1.8 ± 0.1	12.7 ± 0.4
lst8-1	7.4 ± 1.4	35.9 ± 5.8
lst8-6	245.7 ± 16.1	14.6 ± 1.5
lst8-7	90.2 ± 7.6	31.3 ± 2.5
lst8-8	161.2 ± 3.7	20.7 ± 1.6
lst8-9	113.2 ± 13.2	26.3 ± 2.8
lst8-11	181.4 ± 23.7	24.2 ± 3.0
lst8-13	181.7 ± 28.4	21.7 ± 3.0
lst8-15	7.0 ± 1.4	105.8 ± 13.7
lst8-16	183.6 ± 14.2	29.0 ± 5.7

Strains were *ura3-52* (CKY772), *lst8-1 ura3-52* (CKY773), *lst8-6 ura3-52* (CKY774), *lst8-7 ura3-52* (CKY775), *lst8-8 ura3-52* (CKY788), *lst8-9 ura3-52* (CKY789), *lst8-11 ura3-52* (CKY790), *lst8-13 ura3-52* (CKY791), *lst8-15 ura3-52* (CKY792), *and lst8-16 ura3-52* (CKY793), containing pEC261 for P_{CIT2}-*LacZ* or pMS29 for P_{GAP1}-*LacZ*.

in Rtg1/3p but not in Gln3p regulation, whereas the *lst8-1* mutant had a stronger defect in Gln3p but not in Rtg1/3p regulation (Table II and Table III). Unlike rapamycin treatment, which strongly affects both Rtg1/3p and Gln3p simultaneously, the *lst8-1* and *lst8-6* mutations disrupted one branch of the regulation more strongly than the other.

To test whether other *lst8* alleles show genetically distinct interactions with the Rtg1/3p and Gln3p pathways, we isolated additional *lst8* temperature-sensitive alleles by PCR mutagenesis and performed P_{CIT2}-LacZ reporter assays in glutamate and P_{GAP1}-LacZ reporter assays in glutamine. All mutants were sequenced and represent independent alleles with 1–4 missense mutations each (unpublished data). We found that, like *lst8-6*, the *lst8-8, lst8-9, lst8-11, lst8-13,* and *lst8-16* mutants showed strong defects in Rtg1/3p regulation, but only modest defects in Gln3p regulation (Table V). In contrast, the *lst8-15* mutant showed a modest defect in Rtg1/3p regulation, but a stronger defect in Gln3p regulation. Thus, *lst8* alleles appear to differentially affect the Rtg1/3p and Gln3p transcription pathways, suggesting that Lst8p may be the component that transduces the different outputs of the Tor1/2p complex.

Localization of Lst8p

We examined the localization of Lst8p by fractionation of lysates from a strain containing an integrated, fully functional *HA-LST8.* By differential centrifugation, most Lst8p sedimented after centrifugation at both 13,000 *g* and 100,000 *g*, suggesting that Lst8p is membrane associated (Fig. 8 A). To determine conditions for extraction of Lst8p from the insoluble fraction, we treated cell extracts with detergent (1% Triton X-100), high pH, or chaotropic agents such as salt or urea. Lst8p was extracted to the soluble fraction very efficiently by urea and to a lesser extent by high pH or high salt, indicating that Lst8p was peripherally associated with membranes (Fig. 8 B).

To confirm the membrane association of Lst8p, we performed flotation gradients to separate soluble proteins and proteinaceous complexes, which remain at the bottom of the gradient, from membrane-associated proteins, which rise to the isopycnic region of the gradient. Membranes from a crude lysate were collected by centrifugation at 100,000 *g*, layered at the bottom of a continuous 30–50% (wt/wt) sucrose gradient, and centrifuged at 100,000 *g* for 17 h. Membrane-associated Lst8p was found mainly in fractions 3–6, cofractionating with GDPase and Pep12p, the Golgi and endosomal membrane marker proteins (Fig. 8 C). The less dense peak from fractions 5–7 of the trans-Golgi marker Kex2p, which was shown previously to fractionate in two peaks (Cunningham and Wickner, 1989), and the vacuole marker protein Vph1p both partially overlapped with the peak of Lst8p. The large pool of Lst8p in fraction 16, which cofractionated with the cytosolic marker protein Pgk1p (unpublished data), may originate from Lst8p that exists in a large protein complex that sediments at 100,000 *g*. Alternatively, the Lst8p in fraction 16 may have dissociated from membranes during lysate preparation or fractionation. It is unlikely that the Lst8p in fraction 16 represents a plasma membrane localization because Lst8p is still found in fraction 16 in flotation experiments with continuous 20–60% sucrose gradients, in which the plasma membrane is found in fractions 12–15 (unpublished data).

We also used fluorescence microscopy to examine Lst8p localization. A GFP–Lst8p fusion, which complements an *lst8*Δ mutation, was localized to discrete bodies, some adjacent to the vacuole (Fig. 8 D), consistent with the cofractionation of Lst8p with Golgi and endosomal membranes.

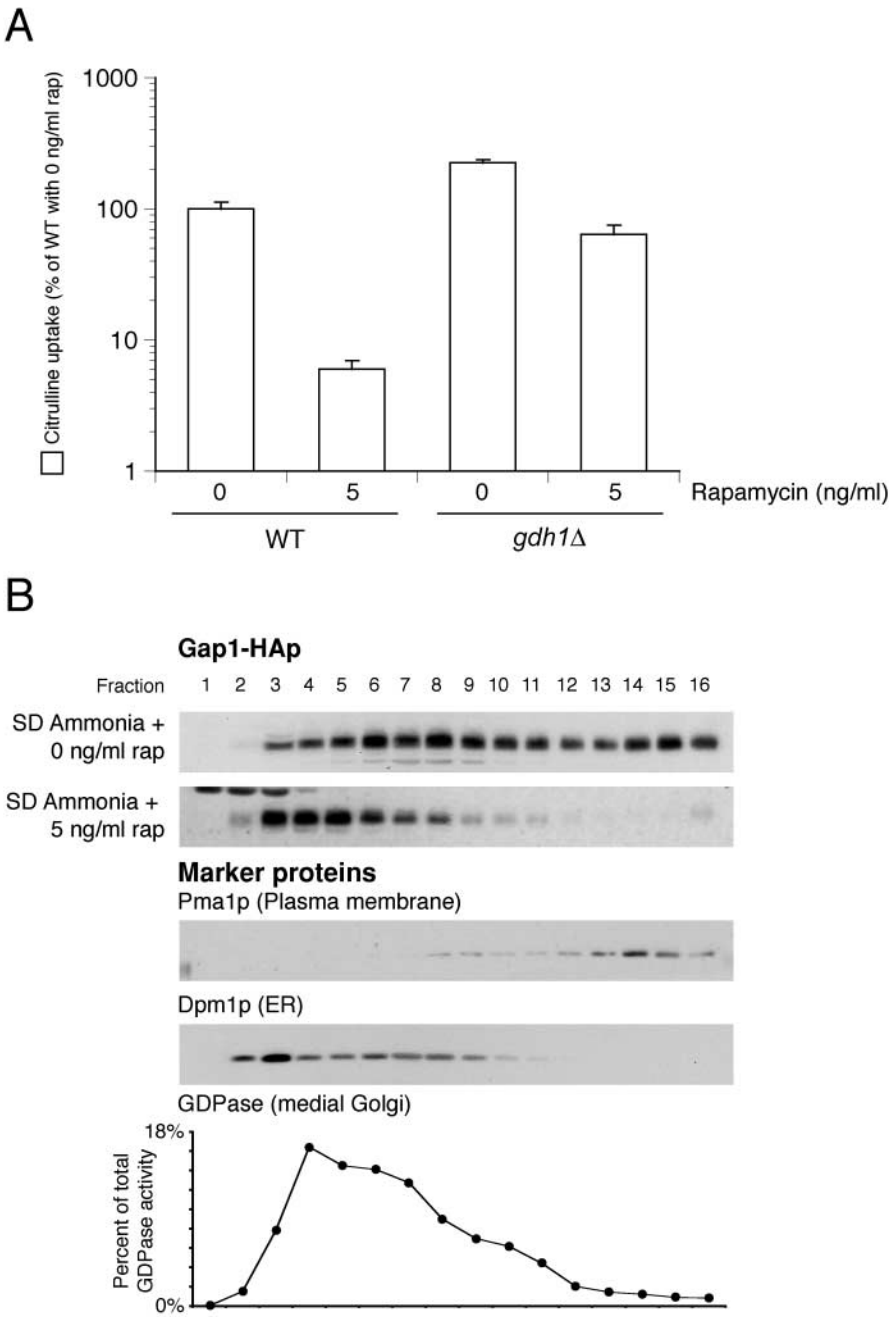

Figure 7. **A sublethal concentration of rapamycin causes a defect in Gap1p sorting to the plasma membrane.** Strains were grown for 18 h to exponential phase in ammonia medium with empty drug vehicle or with 5 ng/ml rapamycin. (A) Gap1p activity was measured by assaying the rate of [^{14}C]citrulline uptake in wild-type (CKY759) and *gdh1*Δ (CKY762), both containing P_{ADH1}-GAP1-HA. The absolute Gap1p activity of wild-type with no rapamycin is 2,864 pmol/min/OD_{600}. (B) The wild-type strain in A was harvested, and cell extracts were subjected to isopycnic fractionation on 20–60% sucrose density gradients. Pma1p, Dpm1p, and GDPase fractionated in a similar manner in the presence or absence of rapamycin.

We performed flotation gradients with a strain coexpressing HA-Lst8p and HA-Tor1p, and found that HA-Lst8p and HA-Tor1p cofractionate with each other (Fig. 8 E). The peak of Tor1p in fractions 3–6 is broader than the Lst8p peak, perhaps indicating that Tor1p is also present on other membranes that lack Lst8p or that more Tor1p dissociated from membranes while floating up through the gradient. Like Lst8p, Tor1p has significant overlap with the endosomal, Golgi, and vacuolar marker proteins Pep12p, GDPase, and Vph1p (Fig. 8 E). We also tested whether the *lst8-1, lst8-6,* and *lst8-7* mutations changed the fractionation pattern of Tor1p in these flotation gradients, but found no significant effect (unpublished data). Thus, Lst8p is a membrane-associated protein that appears to localize to the Golgi or endosomal compartments with Tor1p.

Discussion

In this paper, we provide genetic and biochemical evidence that *LST8* encodes a positively acting component of the TOR pathway. Lst8p associates with Tor1p and Tor2p, and is involved in the regulation of Rtg1/3p and Gln3p, and in maintenance of cell wall integrity. We can now explain the role of Lst8p in the regulation of Gap1p sorting by the three distinct regulatory processes diagramed in Fig. 9: (1) Tor1/2p and Lst8p act together to negatively regulate both the Rtg1/3p and Gln3p transcription factors, limiting the synthesis of α-ketoglutarate, glutamate, and glutamine; (2) inactivation of the TOR pathway by mutation of *LST8* causes an increase in the intracellular pools of glutamate and glutamine, as well as the other amino acids derived from glutamate and glutamine; and (3) because all amino acids can act as a signal to cause

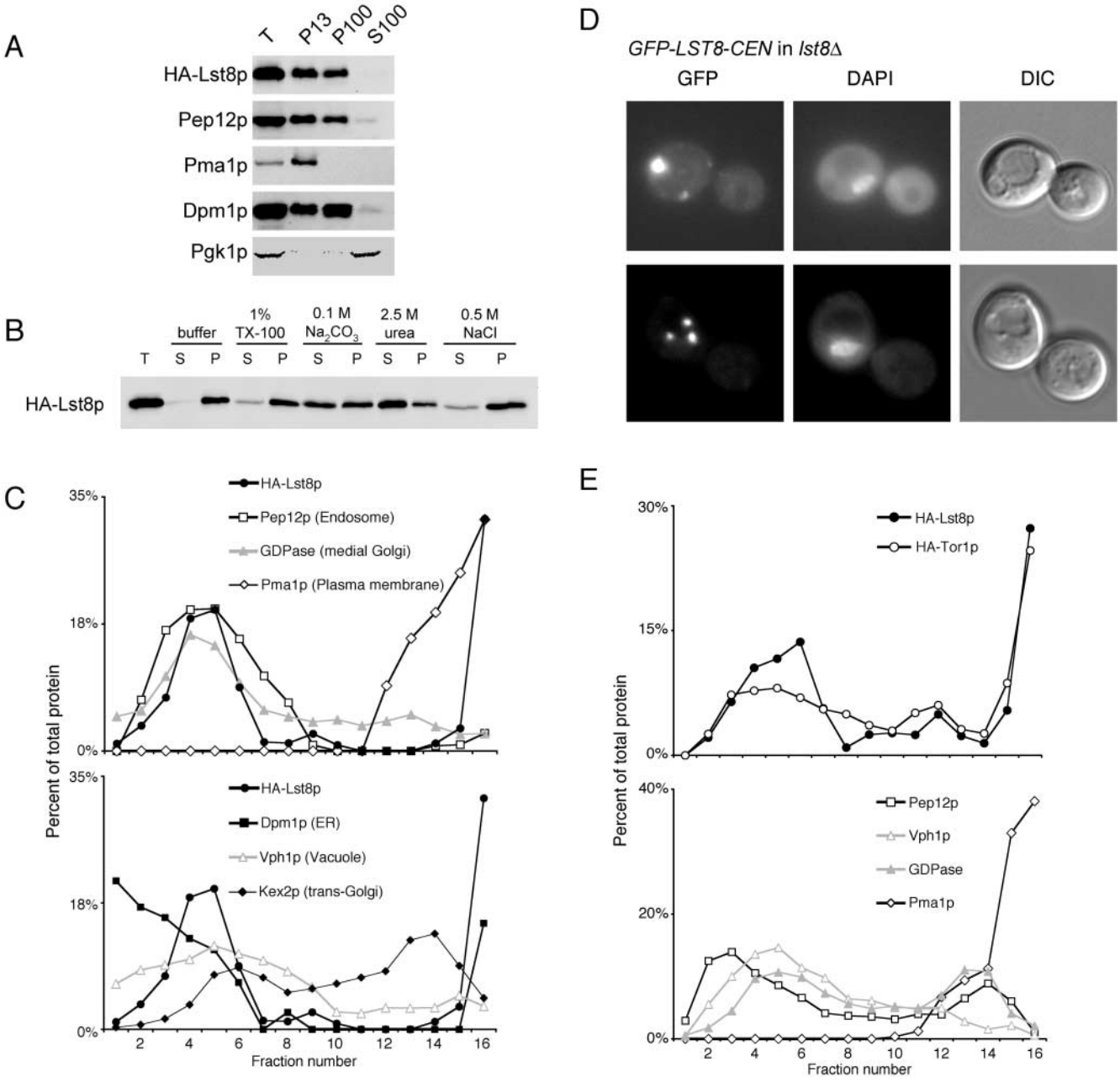

Figure 8. **Lst8p is a peripheral membrane protein that cofractionates with Golgi and endosomal compartments and with Tor1p.** (A) A cleared cell lysate from a strain with integrated *HA-LST8* (CKY784) was fractionated by centrifugation at 13,000 *g*, then at 100,000 *g*. Compartment markers are as follows: Pep12p, endosome; Pma1p, plasma membrane; Dpm1p, ER; and Pgk1p, cytosol. The exposure time for the Pgk1p panel was very short relative to the other panels. (B) A cleared cell lysate from CKY784 was incubated with the treatments shown for 1 h at 4°C, then centrifuged for 1 h at 100,000 *g*. (C) Fractionation of membranes on a flotation gradient shows that HA-Lst8p associates with membranes. A cleared cell lysate from CKY784 was centrifuged at 100,000 *g* for 1 h onto a cushion of 80% (wt/vol) sucrose. The membranes were collected and loaded at the bottom of a continuous 30–50% sucrose gradient, and centrifuged at 100,000 *g* for 17 h. (D) GFP-Lst8p was visualized in an *lst8*Δ strain containing *GFP-LST8* on a centromere plasmid (CKY785). (E) HA-Tor1p–containing membranes cofractionate with HA-Lst8p membranes. Membranes from a strain coexpressing *HA-LST8* and *HA-TOR1* (CKY800) were fractionated as in C.

Gap1p sorting to the vacuole, mutation of *LST8* causes Gap1p to be sorted to the vacuole. As validation of this model, we have shown that growth of wild-type cells in a low, sublethal concentration of rapamycin or that mutation of *LST8* produces an increase in amino acid pools and a consequent decrease in Gap1p activity caused by sorting to the vacuole.

The finding that the cell sorts Gap1p to the vacuole when TOR pathway function is impaired, by a low rapamycin concentration or by mutation of *LST8,* elucidates the different effects of the TOR pathway on *GAP1* transcription and the sorting of Gap1p. Previously, it was proposed that rapamycin treatment would have all of the effects of nitrogen starvation and cause both an increase in *GAP1* transcription and Gap1p sorting to the plasma membrane (Beck et al., 1999). In contrast, here we have shown that rapamycin causes distinct and separable effects on *GAP1* transcription

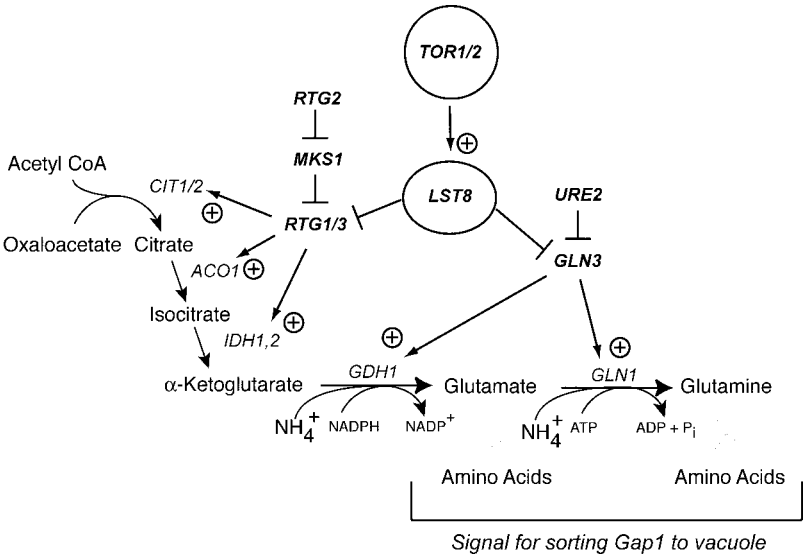

Figure 9. **A model of how the Tor1/2 and Lst8 proteins affect amino acid biosynthesis and Gap1p sorting.** Tor1/2p and Lst8p negatively regulate the activity of the Rtg1p and Rtg3p transcription factors, decreasing the expression of enzymes responsible for α-ketoglutarate synthesis and limiting the synthesis of α-ketoglutarate, glutamate, glutamine, and the other amino acids. Tor1/2p and Lst8p lso negatively regulate the activity of the Gln3p transcription factor, decreasing the expression of *GDH1* and *GLN1*, further limiting amino acid biosynthesis (Mitchell and Magasanik, 1984; Daugherty et al., 1993). Thus, mutation of *lst8* or other impairment of Tor1/2p activity (such as treatment with a sublethal concentration of rapamycin) causes increased amino acid levels, which act as a signal for sorting Gap1p to the vacuole.

and on Gap1p sorting. We show that a partial inactivation of TOR with 5 ng/ml rapamycin causes less Gap1p to be sorted to the plasma membrane, an effect opposite to that of nitrogen starvation. Although the transcriptional induction of genes such as *GAP1* and *CIT2* caused by high levels of rapamycin occurs rapidly (Beck and Hall, 1999; Cardenas et al., 1999; Hardwick et al., 1999; Komeili et al., 2000), the effect of low levels of rapamycin on Gap1p sorting takes place only after several hours (unpublished data). This delayed effect on Gap1p sorting presumably corresponds to the time needed for the cellular amino acid levels to rise through the rapamycin-dependent induction of Rtg1/3p targets such as *CIT2*. Recently, a sublethal concentration of rapamycin (10 ng/ml) was used to inhibit pseudohyphal development in yeast of the Σ1278b background in response to nitrogen limitation (Cutler et al., 2001). For our strains in the S288C background growing on minimal ammonia medium, we found that there was only a narrow range of rapamycin concentrations that were not lethal but that had an effect on Gap1p sorting; below 3 ng/ml, rapamycin had no effect, and at or above 10 ng/ml, rapamycin caused a significant growth defect (unpublished data). The range was narrower still for *gdh1*Δ, which grows more slowly than wild-type on ammonia medium even in the absence of rapamycin.

How do Lst8p and Tor1/2p act together to regulate Rtg1/3p and Gln3p activity? Our analysis of a collection of *lst8* mutants showed that some alleles had a greater effect on Rtg1/3p-dependent transcription than on Gln3p-dependent transcription, whereas other alleles had a greater effect on Gln3p-dependent transcription (Table V). The qualitatively different interactions of Lst8p with these two regulatory pathways imply that Lst8p has two genetically separable functions; apparently different parts of the Lst8 protein interact with either the Rtg1/3p or Gln3p regulatory pathways. This finding suggests that Lst8p is the subunit of the TOR complex that communicates with the downstream effectors of the TOR pathway.

The TOR proteins are thought to have two distinct functions. One function, the integration of nutrient signals with cell growth, can be performed by either Tor1p or Tor2p and is inhibited by rapamycin treatment. The second function, the maintenance of the actin cytoskeleton and cell wall integrity, is unique to Tor2p and is not inhibited by rapamycin (Zheng et al., 1995; Schmelzle and Hall, 2000). Our finding that *lst8* mutants exhibit both the properties of rapamycin-treated cells and the defects in cell wall integrity of *tor2* mutants, and our finding that Lst8p associates with both Tor1p and Tor2p imply that Lst8p acts with the *TOR* gene products to promote both the shared and the Tor2p-unique function. This result is in agreement with the recent report that Lst8p is found in two types of TOR complexes, proposed to fulfill the two different TOR functions (Loewith et al., 2002). However, although Loewith et al. (2002) observed depolarized actin in cells with P_{GAL1}-LST8 after 15 h in glucose, we did not see any actin defects in *lst8* mutants at 24°C in ammonia medium (unpublished data), the same conditions under which we see a strong Gap1p sorting defect. These results suggest that although complete depletion of Lst8p may eventually cause actin depolarization, the Gap1p sorting defect we observe in *lst8* mutants is not due to a cytoskeletal defect.

Rtg2p is a positive regulator of Rtg1/3p activity, but there is conflicting experimental data on the relationship between Tor–Lst8p and Rtg2p in the regulation of Rtg1/3p. Powers and colleagues found that inactivation of the TOR pathway by rapamycin in an *rtg2*Δ mutant fails to induce *CIT2* expression by Rtg1/3p, and concluded that *rtg2*Δ is epistatic to TOR inactivation (Komeili et al., 2000; Dilova et al., 2002). On the other hand, Liu et al. (2001) found that mutation of *lst8* could restore *CIT2* expression to an *rtg2*Δ mutant, and therefore concluded that *lst8* is epistatic to *rtg2*Δ. Like Liu et al., we found that some *lst8* alleles could restore *CIT2* expression in an *rtg2*Δ background (Table II), which again suggested that *lst8* is epistatic to *rtg2*Δ. One possible explanation for the conflicting epistasis results with *rtg2*Δ is that abrupt inactivation of TOR by treatment with rapamycin may have a different effect on the regulatory network controlling Rtg1/3p activity than constitutive inactivation of TOR complex activity by an *lst8* mutation. We considered the possibility that Rtg2p

might be a general negative regulator of Tor–Lst8p, and tested for effects of *rtg2*Δ mutants on Gln3p-dependent transcription, a second output of the Tor–Lst8p pathway. However, we did not observe an effect of a *rtg2*Δ mutation on Gln3p activity using a P_{GAP1}-LacZ reporter on glutamate, indicating that Rtg2p specifically regulates Rtg1/3p. The existing data regarding Rtg2p is compatible with a model in which Rtg2p acts as a negative regulator of Mks1p (Dilova et al., 2002; Sekito et al., 2002), and Rtg2p and Mks1p act in parallel to Tor–Lst8p to regulate Rtg1/3p activity (Fig. 9). Clarification of the precise relationship between Rtg2p and the TOR pathway awaits further biochemical characterization of Rtg2p.

We found that Lst8p is associated with membranes and appears to localize to the endosomal/Golgi compartments. Tor1p also cofractionates with Lst8p and with endosomal/Golgi and vacuolar markers (Fig. 8). Previous studies of Tor1/2p localization have led to a variety of conclusions about the identity of the membranes with which Tor is associated: Cardenas and Heitman (1995) reported that Tor2p associates with vacuolar membranes and Kunz et al. (2000) reported that Tor1p and Tor2p associate with the plasma membrane and with a second, unidentified membrane compartment. Recently, Loewith et al. (2002) reported that a pool of Lst8p eluted separately from the Tor proteins during gel filtration of a lysate prepared by agitation with glass

Table VI. **Strains (all are isogenic with S288C). All are from this paper unless noted**

Strain	Genotype	Source
CKY8	MATα *ura3-52 leu2-3, 112*	Kaiser strain collection
CKY443	MATa prototroph	Kaiser strain collection
CKY445	MATα *gap1Δ::LEU2 leu2-3, 112*	Kaiser strain collection
CKY526	MATa *lst8-1*	Kaiser strain collection
CKY758	MATa *mks1Δ::kanMX6*	Kaiser strain collection
CKY759	MATα P_{ADH1}-*GAP1-HA*	Kaiser strain collection
CKY762	MATa *gdh1Δ::kanMX6* P_{ADH1}-*GAP1-HA*	Kaiser strain collection
CKY768	MATα *lst8-1* P_{ADH1}-*GAP1-HA*	
CKY769	MATα *lst8-1 gdh1Δ::kanMX6* P_{ADH1}-*GAP1-HA*	
CKY770	MATa *lst8-6*	
CKY771	MATa *lst8-7*	
CKY772	MATa *ura3-52*	
CKY773	MATa *lst8-1 ura3-52*	
CKY774	MATa *lst8-6 ura3-52*	
CKY775	MATa *lst8-7 ura3-52*	
CKY776	MATa *mks1Δ::kanMX6 ura3-52*	
CKY777	MATa *ure2Δ::kanMX6 ura3-52*	
CKY778	MATa *gln3Δ::kanMX6*	
CKY779	MATa *GLN3-13xmyc-kanMX6*	
CKY780	MATa *lst8-1 GLN3-13xmyc-kanMX6*	
CKY781	MATa *lst8-7 GLN3-13xmyc-kanMX6*	
CKY782	MATa *lst8-7 gln3Δ::URA3 ura3-52*	
CKY783	MATa *LST8-6xmyc ura3-52*	
CKY784	MATα *3xHA-LST8*	
CKY785	MATα *lst8Δ::HIS3 ura3-52 leu2-3, 112 his3Δ200* [pEC264]	
CKY786	MATa *lst8-6 cwh41Δ::kanMX4 lys2Δ0 ura3Δ0*	
CKY787	MATa *lst8-6 fks1Δ::kanMX4*	
CKY788	MATa *lst8-8 ura3-52*	
CKY789	MATa *lst8-9 ura3-52*	
CKY790	MATa *lst8-11 ura3-52*	
CKY791	MATa *lst8-13 ura3-52*	
CKY792	MATa *lst8-15 ura3-52*	
CKY793	MATa *lst8-16 ura3-52*	
CKY794	MATa *tor1Δ::kanMX4 ura3-52*	
CKY795	MATα *rtg2Δ::kanMX6 ura3-52*	
CKY796	MATa *rtg2Δ::kanMX6 lst8-1 ura3-52*	
CKY797	MATa *rtg2Δ::kanMX6 lst8-6 ura3-52*	
CKY798	MATa *rtg2Δ::kanMX6 lst8-7 ura3-52*	
CKY799	MATα *rtg2Δ::kanMX6 mks1Δ::kanMX6 ura3-52*	
CKY800	MATa *tor1Δ::kanMX4 HA-LST8 ura3-52* [pEC267]	
Y16864	MATα *tor1Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf collection
Y14395	MATα *cwh41::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf collection
Y15251	MATα *fks1Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf collection

beads, suggesting that not all the Lst8p is associated with Tor1/2p or that some Lst8p dissociated from Tor1/2p during lysate preparation. Kog1p/mRaptor is also a TOR-associated protein with WD-repeats whose association with TOR is sensitive to nutrient conditions (Hara et al., 2002; Kim et al., 2002). Thus, Lst8p may act with Tor1/2p and Kog1p/Raptor as a component of a large complex on endosomal/Golgi membranes for sensing intracellular nutrients and signaling to metabolic pathways.

Materials and methods

Strains, plasmids, and media

The yeast strains used in this work (listed in Table VI) are all in the S288C background. One characteristic of the S288C background is high Gap1p and Put4p activity when ammonia is used as a nitrogen source (Courchesne and Magasanik, 1983). The *lst8Δ::HIS3* allele was made using the fusion PCR protocol from the Botstein lab. Complete gene deletions of *GLN3*, *URE2*, and *RTG2* were constructed by gene replacement with the *kanMX6* cassette by homologous recombination (Wach et al., 1994). Tagging of *GLN3* with 13x*myc* at its COOH terminus was performed by homologous recombination (Longtine et al., 1998). Yeast strains containing *HA-LST8* and *LST8-6xmyc* were constructed as follows: plasmid pEC36 contained *LST8* flanked by 361 bp of the 5′ region and 412 bp of the 3′ region in pRS315. Unique NotI restriction sites were inserted either just after start or just before stop by site-directed mutagenesis (creating pEC62 and pEC63), and *3xHA* or *3xmyc* was inserted creating pEC85 (*HA-Lst8*) or pEC70 (*Lst8-6xmyc*), respectively. Constructs were verified by sequencing and by complementation of *lst8Δ*. The *LST8*-containing inserts from pEC85 and pEC70 were moved into pRS306 and integrated into CKY772 by two-step gene replacement. Plasmids used in this work were as follows: pMS29, a P_{GAP1}-LacZ fusion at codon 53 of *GAP1* in a *URA3-CEN* vector (Stanbrough and Magasanik, 1995); pEC261, a P_{CIT2}-LacZ fusion after codon 3 of *CIT2* in pRS316; pEC262, *TOR1* in pRS316; pEC263, *TOR2* in pRS316; pEC267, *HA-TOR1* in pRS316; pEC268, *HA-TOR2* in pRS316; and pEC264, *sGFP-LST8* in pRS315. Plasmid pEC261 was constructed by ligating a PCR fragment containing 390 bp of the *CIT2* promoter (that includes the region required for the transcriptional regulation of *CIT2*; Liao and Butow, 1993) and the first three codons of the *CIT2* gene, to the *LacZ* coding sequence in pRS316. Plasmids pEC262 and pEC263 were constructed by gap repair in yeast of cut plasmids containing nucleotides −499 to −6 of the 5′ region and +1 to +361 of the 3′ region for *TOR1*, or nucleotides −522 to −4 of the 5′ region and +20 to +373 of the 3′ region for *TOR2*. To construct pEC267 and pEC268, unique restriction sites were inserted after the start codons of *TOR1* and of *TOR2*, and *3xHA* was inserted by homologous recombination. To construct pEC264, *sGFP* was ligated into the unique NotI site just after the start codon of *LST8* in pEC62 (described above). Minimal media containing ammonia, glutamate, or glutamine as a nitrogen source were prepared as described previously (Roberg et al., 1997b). Supplemented minimal medium (SMM) was prepared as described previously (Adams et al., 1996).

Isolation and integration of *lst8* temperature-sensitive alleles

PCR mutagenesis of the *LST8* gene was performed as described previously (Muhlrad et al., 1992). In brief, the region from 400 bp 5′ to *LST8* to 247 bp 3′ to *LST8* was amplified using Taq in the presence of 0.25 mM $MnCl_2$, and one-fifth the normal concentration of dATP. The PCR fragment and pEC36 (described above) cut with BsmI were transformed into ECY269 that is *lst8Δ::HIS3 ura3-52 leu2-3, 112 his3Δ200* [*LST8* in pRS316]. For the *lst8-6* allele, pEC36 was mutagenized with hydroxylamine as described previously (Adams et al., 1996) and transformed into ECY269. Transformants on SMM-leucine plates were replica-plated onto plates with 5-fluoroorotic acid, then screened for temperature sensitivity. Plasmids that conferred temperature-sensitive growth were tested by retransformation. Inserts were ligated into pRS306 for integration at the *LST8* locus by two-step gene replacement.

Assays for amino acid uptake, β-galactosidase, and total amino acid content

Strains were cultured to 4–8 × 10^6 cells/ml, washed twice with nitrogen-free medium by filtration on a 0.45-μm nitrocellulose filter (Millipore), and amino acid uptake assays were performed as described previously (Roberg et al., 1997b). β-Galactosidase activity was measured with the permeabilized cell method (Adams et al., 1996). Two independent transformants were grown at RT (22°C) and assayed in duplicate. Each experiment was performed 2–5 times with similar results. Total amino acid analysis was performed as described previously (Chen and Kaiser, 2002).

Immunofluorescence and fluorescence microscopy

Immunofluorescence was performed using standard protocols (Adams et al., 1996) with the following modifications. PBS + 2% BSA was used for blocking and for diluting antibodies, cells were incubated with primary antibody overnight at 4°C, and samples were washed 15 times after each antibody incubation. Antibodies used were purified monoclonal 9E10 (Zymed Laboratories) and Alexa® 488–conjugated goat anti–mouse IgG (Molecular Probes, Inc.). For GFP microscopy, cells were grown in SMM-leucine media overnight to exponential phase, then Tris-HCl, pH 8.0 was added to 100 mM and NaN_3 was added to 1% for 15 min before viewing to ensure that GFP was folded and to enhance detection of GFP in acidic compartments (Bilodeau et al., 2002). Images were collected using a fluorescence microscope (Eclipse E800; Nikon), a digital camera (Hamamatsu Corporation), and Openlab software (Improvision).

Equilibrium density centrifugation, differential centrifugation, and extraction of proteins from the particulate fraction

Protocols are described in Kaiser et al. (2002). For the differential centrifugation and the extraction of proteins from the particulate fraction protocols, cells were lysed by spheroplasting and douncing. Antibodies used were: mouse anti-HA 16B12 (Covance); mouse anti-HA 12CA5 (BAbCo); rabbit anti-Pma1p (a gift of S. Losko and R. Kölling, Heinrich-Heine-Universitat, Düsseldorf, Germany), mouse anti-Dpm1p (Molecular Probes, Inc.); mouse anti-Pgk1p (Molecular Probes, Inc.); mouse anti-Vph1p (Molecular Probes, Inc.); and rabbit anti-Pep12p. Anti-Pep12p serum was made using a standard antibody protocol (Covance) with 6xHis-Pep12p made from truncated *PEP12* in pET24a (a gift of M. Lewis and H. Pelham, MRC Laboratory of Molecular Biology, Cambridge, UK).

Flotation gradient with Lst8p or Tor1p-containing membranes

Flotation gradients were performed as described previously (Kaiser et al., 2002), with the following modifications: 3 × 10^9 cells from a logarithmically growing culture were harvested by filtration, then washed in ice-cold de-energizing buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaN_3, and 10 mM KF), and washed in ice-cold STE10 (10% wt/wt sucrose, 10 mM Tris-HCl, pH 7.5, and 10 mM EDTA, pH 8.0) by centrifugation. Cells were lysed by agitation with glass beads in 0.75 ml lysis buffer (STE10 with PMSF and pepstatin). 2.25 ml lysis buffer was added, and the lysate was cleared by centrifugation at 500 *g* for 3 min. Membranes were collected by layering 2 ml of the cleared lysate onto a cushion of 0.2 ml STE80 (80% wt/vol sucrose, 10 mM Tris-HCl, pH 7.5, and 10 mM EDTA) and centrifuging in a TLS-55 rotor (Beckman Coulter) at 100,000 *g* for 1 h at 4°C. Membranes that collected at the interface were combined with enough STE80 to make the density of the solution equivalent to the density of STE50 (50% wt/wt sucrose, 10 mM Tris-HCl pH 7.5, and 10 mM EDTA). A volume of membrane solution corresponding to 3 × 10^8 cells was loaded at the bottom of a 30–50% (wt/wt) continuous sucrose gradient and centrifuged at 100,000 *g* for 17 h with no brake in an SW55Ti rotor (Beckman Coulter). Fractions were collected manually from the top of the gradient. A portion of each fraction was subject to TCA precipitation, SDS-PAGE, and Western blotting, or was assayed for GDPase and Kex2p activity as described previously (Kaiser et al., 2002). Intensity of protein bands on Western blots was quantitated using the Kodak Image Station 440 imaging system and Kodak 1D software (PerkinElmer).

Immunoprecipitation and immunoblotting of Lst8-associated proteins

Cells (10^8) growing logarithmically in SMM-uracil medium were harvested and washed with ice-cold 50 mM Hepes, pH 7.5, and 10 mM NaN_3. Cells were lysed by agitation with glass beads in Co-IP buffer (20 mM Hepes, pH 6.8, 80 mM potassium acetate, 5 mM magnesium acetate, and 0.5% CHAPS) with protease inhibitors (1 mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 2 μg/ml aprotinin), then the lysate was diluted to 1 ml with Co-IP buffer with protease inhibitors. The lysate was cleared by centrifugation at 13,000 *g* for 3 min, then the supernatant was precleared by incubation with protein A Sepharose for 30 min at 4°C. A portion of the precleared lysate was removed as the "total" sample. To the remaining lysate, rabbit anti-myc antibody (9E10, Santa Cruz Biotechnology) was added and incubated for 2 h at 4°C. Then, Protein A Sepharose was added

and the mixture incubated for 1 h at 4°C. Immunoprecipitates were washed three times with Co-IP buffer + 0.1% CHAPS, and once with detergent-free Co-IP buffer. Immunoprecipitates were solubilized by incubation in sample buffer for 30 min at 37°C and resolved by SDS-PAGE. Antibodies used for immunoblotting were mouse anti-HA 12CA5 and mouse anti-myc 9E10 (Covance).

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Note added in proof. In a recent report, T. Powers and colleagues (Wedaman, K.P., A. Reinke, S. Anderson, J. Yates 3rd, J.M. McCaffery, and T. Powers. 2003. *Mol. Biol. Cell.* 14:1204–1220) used immunogold electron microscopy to colocalize Lst8p and Tor2p to punctate, membranous sites adjacent to (but distinct from) the plasma membrane and other sites within the cell. This localization of Tor/Lst8 is consistent with the endosomal/Golgi localization for Lst8p based on membrane fractionation we report in this paper.

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