LSH8 negatively regulates amino acid biosynthesis as a component of the TOR pathway

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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 glucose 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...
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 \( \alpha \)-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 \( \alpha \)-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\( \Delta \) 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-
tand was shown to greatly diminish sorting of Gap1\(p\) 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 transcript and decreased sensitivity to glutamate for CIT2 repression (Liu et al., 2001). These findings suggested that either Lst8p has an indirect effect on Gap1\(p\) sorting, like Mks1\(p\), 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 Gap1\(p\) permease sorting and find that the effects of \(lst8\) mutations on Gap1\(p\) 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 Gap1\(p\) sorting is indirect

An \(lst8-1\) mutation causes Gap1\(p\) to be sorted to the vacuole under growth conditions in which Gap1\(p\) is normally sorted to the plasma membrane (Roberg et al., 1997a). Because Gap1\(p\) 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 Gap1\(p\) activity was an effect on Gap1\(p\) 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 Gap1\(p\) activity as measured by uptake of [\(^{14}\)C]citrulline, which is transported exclusively by Gap1\(p\) (Fig. 1A), and no Gap1\(p\) localized to the plasma membrane (Fig. 1B). Uptake of [\(^{14}\)C]arginine, which is transported by Gap1\(p\) and by the arginine permease Can1\(p\), is similar in the \(lst8-1\) and \(gap1\) \(\Delta\) mutants, indicating that the effect of \(lst8-1\) on permease sorting is specific for Gap1\(p\), and is not the result of a general decrease in all permease activity (Fig. 1A).

Butow and colleagues recently reported that Lst8p negatively regulates the Rtg1/3 and Rtg3/3 transcription factors, which control the transcription of genes responsible for \(\alpha\)-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 \(\alpha\)-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; Komelj et al., 2000). Deletion of \(MKS1\), another negative regulator of Rtg1/3p, has been shown to result in elevated intracellular levels of \(\alpha\)-ketoglutarate, glutamate, and total amino acids, and as a consequence leads to decreased sorting of Gap1\(p\) to the plasma membrane (Feller et al., 1997; Chen and Kaiser, 2002). To determine if the \(lst8-1\) mutation had an effect similar to \(mks1\) \(\Delta\) on cellular amino acid content, we performed whole-cell amino acid analysis and found that \(lst8-1\), like \(mks1\) \(\Delta\), had \(\sim 1.5\)–\(2.5\)-fold higher intracellular levels of glutamate and total amino acids than wild-type cells (Table I).

If an \(lst8-1\) mutation causes a Gap1\(p\) sorting defect indirectly by de-repressing Rtg1/3 and increasing cellular glutamate levels, then the elimination of \(GDH1\), which encodes the major enzyme for the synthesis of glutamate from \(\alpha\)-ketoglutarate on ammonia medium (Grenson et al., 1974), should significantly diminish the Gap1\(p\) sorting defect of an \(lst8-1\) mutant. We found that the \(lst8-1\) \(gdh1\) \(\Delta\) double mutant had 30% of wild-type Gap1\(p\) activity, compared with <1% for the \(lst8-1\) mutant alone during growth on ammonia (Fig. 1A). The \(lst8-1\) \(gdh1\) \(\Delta\) double mutant also showed more Gap1\(p\) sorted to the plasma membrane than an \(lst8-1\) mutant (Fig. 1B). Together with the amino acid analysis of \(lst8-1\), these data suggested that the Gap1\(p\) 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) | WT | \(lst8-1\) | \(mks1\) \(\Delta\) | WT + 5 ng/ml rapamycin |
|--------------------------------------|----|------------|----------------|------------------------|
| Glt                                  | 1.0| 2.4 ± 0.1  | 2.4 ± 0.2      | 2.5 ± 0.3              |
| Gln                                  | 1.0| 1.9 ± 0.1  | 1.3 ± 0.1      | 4.5 ± 0.1              |
| Glt + Gln                            | 1.0| 2.1 ± 0.1  | 1.8 ± 0.1      | 6.6 ± 0.0              |
| Total amino acids                    | 1.0| 1.7 ± 0.1  | 1.5 ± 0.1      | 2.6 ± 0.0              |

Amino acid contents of \(lst8-1\) and \(mks1\) \(\Delta\) 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 \(mks1\) \(\Delta\) and WT + 5 ng/ml rapamycin. Wild-type (CKY443), \(lst8-1\) (CKY526), and \(mks1\) \(\Delta\) (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.
and *lst8*—7 temperature-sensitive alleles were generated by hydroxyamine 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 ammonium medium at 24°C (unpublished data).

We first checked *lst8*-6 and *lst8*-7 for derepressed Rtg1/3p activity by using a *P*$_{GTP}$-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*$_{GTP}$-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 *rg2*Δ somewhat with regard to *P*$_{GTP}$-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 *rg2*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 glucose (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-

| Strain | *P*$_{GTP}$-LacZ expression on glutamate medium | Standard deviation |
|--------|-----------------------------------------------|-------------------|
|        | (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               |
| mks1Δ  | 729.1                                         | 39.7              |
| ure2Δ  | 4.2                                           | 1.5               |
| rtg2Δ  | 0.8                                           | 0.1               |
| rtg2Δ lst8-1 | 1.4                                    | 0.8               |
| rtg2Δ lst8-6 | 147.8                              | 13.9              |
| rtg2Δ lst8-7 | 33.7                                      | 2.4               |
| rtg2Δ mks1Δ | 752.4                                    | 80.4              |

Strains were *ura3*-52 (CKY772), *lst8*-1 *ura3*-52 (CKY773), *lst8*-6 *ura3*-52 (CKY774), *lst8*-7 *ura3*-52 (CKY775), *mks1Δ* *ura3*-52 (CKY776), *ure2Δ* *ura3*-52 (CKY777), *rtg2Δ* *ura3*-52 (CKY795), *rtg2Δ lst8-1* *ura3*-52 (CKY796), *rtg2Δ lst8-6* *ura3*-52 (CKY797), *rtg2Δ lst8-7* *ura3*-52 (CKY798), and *rtg2Δ mks1Δ* *ura3*-52 (CKY799) containing pEC261.

| Strain | *P*$_{GAP1}$-LacZ expression in glutamine medium | Standard deviation |
|--------|-----------------------------------------------|-------------------|
|        | (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               |
| mks1Δ  | 0.4                                           | 0.1               |
| ure2Δ  | 361.4                                         | 78.9              |
| tor1Δ  | 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.

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**Figure 2. Mutations in lst8 confer rapamycin hypersensitivity.** Wild-type (CKY443), *lst8*-1 (CKY526), *lst8*-6 (CKY770), *lst8*-7 (CKY771), *tor1*Δ (Euroscarf), and *gln3Δ* (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 *gln3Δ* strain is included as an example of a rapamycin-resistant strain.
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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 $P_{\text{GAP1}}$-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 tor1Δ showed a twofold increase in $P_{\text{GAP1}}$-LacZ activity, and wild-type cells treated with rapamycin for 2 h showed a 6.5-fold increase in $P_{\text{GAP1}}$-LacZ activity. In a ure2Δ mutant in which the negative regulation of the Gln3p transcription factor by glutamine has been completely abolished, GAP1 expression was derepressed $\sim$30-fold (Table III). Unlike the nks1Δ and ure2Δ 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 relocation 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.
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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; Hellmwell 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 lst8Δ mutant (unpublished data), indicating that the lst8Δ mutant fails to grow for reasons other than cell wall instability. We found that SDS in the growth medium or fks1Δ or cwh41Δ 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 tor1Δ strain (for HA-TOR1) and complementation of the lethality of a tor2Δ 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.

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 Pad1-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 Pad1-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-
ammonia 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 gdh1Δ (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 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, cwh41Δ (Euroscarf), fks1Δ (Euroscarf), lst8-6 cwh41Δ (CKY786), and lst8-6 fks1Δ (CKY787) were streaked onto YPD and incubated at 24°C or 34°C for 3 or 2 d, respectively.
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 PCTP-LacZ reporter assays in glutamate and PCAPI-LacZ reporter assays in glutamine. All mutants were sequenced and represent independently allelic mutants 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. 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.

![Image](https://example.com/image.png)
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...
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.
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 GAL1 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 S1278b 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 gdh1Δ, 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 Lst8p 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; Schmelze 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 PGLU1-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 rgl2Δ mutant fails to induce CIT2 expression by Rtg1/3p, and concluded that rgl2Δ 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 rgl2Δ mutant, and therefore concluded that lst8 is epistatic to rgl2Δ. Like Liu et al., we found that some lst8 alleles could restore CIT2 expression in an rgl2Δ background (Table II), which again suggested that lst8 is epistatic to rgl2Δ. One possible explanation for the conflicting epistasis results with rgl2Δ 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 rtg2Δ mutants on Gln3p-dependent transcription, a second output of the Tor–Lst8p pathway. However, we did not observe an effect of a rtg2Δ mutation on Gln3p activity using a $P_{\text{GAP1}}$-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   | MATa ura3-52 leu2-3, 112 | Kaiser strain collection |
| CKY443 | MATa prototroph | Kaiser strain collection |
| CKY445 | MATa gap1Δ::LEU2 leu2-3, 112 | Kaiser strain collection |
| CKY526 | MATa lst8-1 | Kaiser strain collection |
| CKY758 | MATa mks1Δ::kanMX6 | Kaiser strain collection |
| CKY759 | MATa $P_{\text{GAP1}}$-GAP1-HA | Kaiser strain collection |
| CKY760 | MATa gdh1Δ::kanMX6 $P_{\text{GAP1}}$-GAP1-HA | Kaiser strain collection |
| CKY761 | MATa lst8-1 | Kaiser strain collection |
| CKY771 | MATa lst8-7 | Kaiser strain collection |
| CKY772 | MATa ura3-52 | Kaiser strain collection |
| CKY773 | MATa lst8-1 ura3-52 | Kaiser strain collection |
| CKY774 | MATa lst8-6 ura3-52 | Kaiser strain collection |
| CKY775 | MATa lst8-7 ura3-52 | Kaiser strain collection |
| CKY776 | MATa mks1Δ::kanMX6 ura3-52 | Kaiser strain collection |
| CKY777 | MATa ure2Δ::kanMX6 ura3-52 | Kaiser strain collection |
| CKY778 | MATa gln3Δ::kanMX6 | Kaiser strain collection |
| CKY779 | MATa GLN3-13xmyc-kanMX6 | Kaiser strain collection |
| CKY780 | MATa lst8-1 GLN3-13xmyc-kanMX6 | Kaiser strain collection |
| CKY781 | MATa lst8-7 GLN3-13xmyc-kanMX6 | Kaiser strain collection |
| CKY782 | MATa lst8-7 gln3Δ::URA3 ura3-52 | Kaiser strain collection |
| CKY783 | MATa LST8-6xmyc ura3-52 | Kaiser strain collection |
| CKY784 | MATa 3xHA-LST8 | Kaiser strain collection |
| CKY785 | MATa lst8Δ::His3 ura3-52 leu2-3, 112 his3Δ200 [pEC264] | Kaiser strain collection |
| CKY786 | MATa lst8-6 cwh41Δ::kanMX4 lys2Δ0 ura3Δ0 | Kaiser strain collection |
| CKY787 | MATa lst8-6 his3Δ::kanMX4 | Kaiser strain collection |
| CKY788 | MATa lst8-8 ura3-52 | Kaiser strain collection |
| CKY789 | MATa lst8-9 ura3-52 | Kaiser strain collection |
| CKY790 | MATa lst8-11 ura3-52 | Kaiser strain collection |
| CKY791 | MATa lst8-13 ura3-52 | Kaiser strain collection |
| CKY792 | MATa lst8-15 ura3-52 | Kaiser strain collection |
| CKY793 | MATa lst8-16 ura3-52 | Kaiser strain collection |
| CKY794 | MATa tor1Δ::kanMX4 ura3-52 | Kaiser strain collection |
| CKY795 | MATa tor1Δ::kanMX4 ura3-52 | Kaiser strain collection |
| CKY796 | MATa tor2Δ::kanMX6 lst8-1 ura3-52 | Kaiser strain collection |
| CKY797 | MATa tor2Δ::kanMX6 lst8-6 ura3-52 | Kaiser strain collection |
| CKY798 | MATa tor2Δ::kanMX6 lst8-7 ura3-52 | Kaiser strain collection |
| CKY799 | MATa tor2Δ::kanMX6 mks1Δ::kanMX6 ura3-52 | Euroscarf collection |
| CKY800 | MATa tor1Δ::kanMX4 HA-LST8 ura3-52 [pEC267] | Euroscarf collection |
| Y16864 | MATa tor1Δ::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf collection |
| Y14395 | MATa cwh41::kanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Euroscarf collection |
| Y15251 | MATa his3Δ1::kanMX4 ura3Δ0 | Euroscarf collection |
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 Ist8Δ::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 13xmyc 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 pCE36 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 the start or just before stop by site-directed mutagenesis (creating pCE62 and pCE63), and 3xHA or 3xmyc was inserted creating pCE85 (HA-Lst8) or pCE70 (LST8-6xmyc), respectively. Constructs were verified by sequencing and by complementation of Ist8Δ. The LST8-containing inserts from pCE85 and pCE70 were moved into pRS306 and integrated into CYC772 by two-step gene replacement. Plasmids used in this work were as follows: pMS29, a pCvector-LacZ fusion at codon 53 of GAP1 in a URA3-CEN vector (Stanbrough and Magasanik, 1995); pCE261, a pCvector-LacZ fusion after codon 3 of CIT2 in pRS316; pCE262, TOR1 in pRS316; pCE263, TOR2 in pRS316; pCE267, HA-TOR1 in pRS316; pCE268, HA-TOR2 in pRS316; and pCE264, 4xGF-LS78 in pRS315. Plasmid pCE261 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; Lio and Butow, 1993) and the first three codons of the CIT2 gene, to the LacZ coding sequence in pRS316. Plasmids pCE262 and pCE263 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 pCE267 and pCE268, unique restriction sites were inserted either just after the start or codons of TOR1 and of TOR2, and 3xHA was inserted by homologous recombination. To construct pCE264, 4xGFP was ligated into the unique NotI site just after the start codon of LST8 in pCE62 (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 Lst8p temperature-sensitive alleles

PCR and integration 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 MnCl₂, and one-fifth the normal concentration of dATP. The PCR fragment and pCE36 (described above) cut with BsmI were transformed into ECY269 that is Lst8Δ::HIS3 ura3-52 leu2-3, 112 his312-200 (LST8 in pRS316). For the Lst8Δ allele, pCE36 was mutagenized with hydroxyamine as described previously (Adams et al., 1996) and transformed into ECY269. Transformants on MM-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 × 10⁶ 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–mouse IgG (Molecular Probes, Inc.). For GFP microscopy, cells were grown in SMN-Leucine media overnight to exponential phase, then Tris-HCl, pH 8.0 was added to 100 mM and NaN₃ 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 extraction of proteins from the particulate fraction protocols, cells were lysed by spheroplasting and douncing. Antibodies used were: mouse anti-HA 116B12 (Covance); mouse anti-δ2CAS (BABCO); rabbit anti-Ptn1p (gift of S. Losko and R. Kölling, Heinrich-Heine-Universität, Düsseldorf, mouse anti-Dnm1p; mouse anti-Lst8p (Molecular Probes, Inc.); mouse anti-Pgk1p (Molecular Probes, Inc.); and rabbit anti-Pep12p. Anti-Pep12p serum was made using a standard antibody protocol (Covance) with 6xHis-Pep12p made from truncated Pep12p in pET2a (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 × 10⁶ 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 Na₂SO₄, and 10 mM KF), and washed in ice-cold STE10 (10% wt/vol 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 1 h at 1°C. Membranes that collected at the interface were combined with enough STE80 to make the density of the solution equivalent to that of STE10 (50% wt/vol sucrose, 10 mM Tris-HCl pH 7.5, and 10 mM EDTA). A volume of membrane solution corresponding to 3 × 10⁶ 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).
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 (Weda-
man, 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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