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RTG-dependent mitochondria to nucleus signaling is negatively regulated by the seven WD-repeat protein Lst8p

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In cells with reduced mitochondrial function, *RTG1*, *2* and *3* are required for expression of genes involved in glutamate synthesis. Glutamate negatively regulates *RTG*-dependent gene expression upstream of Rtg2p, which, in turn, acts upstream of the bHLH/Zip transcription factors, Rtg1p and Rtg3p. Here we report that some mutations [*lst8-(2–5)*] in *LST8*, an essential gene encoding a seven WD40-repeat protein required for targeting of amino acid permeases (AAPs) to the plasma membrane, bypass the requirement for Rtg2p and abolish glutamate repression of *RTG*-dependent gene expression. The *lst8-1* mutation, however, which reduces plasma membrane expression of AAP, cannot bypass the Rtg2p requirement, but still suppresses glutamate repression of *RTG* target gene expression. We show that Lst8p negatively regulates *RTG* gene function, acting at two sites, one upstream of Rtg2p, affecting glutamate repression of *RTG*-dependent gene expression through Ssy1p, an AAP-like sensor of external amino acids, and the other between Rtg2p and Rtg1p–Rtg3p. These data, together with genome-wide transcription profiling, reveal pathways regulated by glutamate, and provide insight into the regulation of cellular responses to mitochondrial dysfunction.

Keywords: LST8/mitochondria/*RTG* genes/WD40 repeat/yeast

Introduction

In the budding yeast, *Saccharomyces cerevisiae*, mitochondrial dysfunction, such as respiratory deficiency or blocks in the tricarboxylic acid (TCA) cycle, results in increased expression of a subset of nuclear genes involved in metabolism, small molecule transport pathways, peroxisomal biogenesis and stress responses (Liao *et al*., 1991; Chelstowska and Butow, 1995; Traven *et al*., 2001; Epstein *et al*., 2001b). This pathway of inter-organelle communication, called retrograde regulation, accommodates cells to their mitochondrial defect. For example, many of the reactions catalyzed by target genes in the retrograde pathway serve, directly or indirectly, to maintain the supply of key TCA cycle intermediates, e.g. oxaloacetate, that would otherwise become severely limiting in cells with mitochondrial deficiencies. The expression of some of the genes in the retrograde pathway, such as *CIT2* (encoding a glyoxylate cycle isoform of citrate synthase), *DLD3* (encoding a D-lactate dehydrogenase) and *PDH1* (a gene involved in propionate metabolism) is directly dependent on three regulatory genes, *RTG1*, *RTG2* and *RTG3* (Liao and Butow, 1993; Jia *et al*., 1997; Chelstowska *et al*., 1999).

In cells that are respiratory deficient or have reduced respiratory activity, the *RTG* genes also regulate the expression of *CIT1*, *ACO1*, *IDH1* and *IDH2* (Liu and Butow, 1999), which encode enzymes catalyzing the first three steps of the TCA cycle that lead to the synthesis of α-ketoglutarate, the precursor of glutamate. In cells with robust mitochondrial respiratory activity, expression of those TCA cycle genes is under the control of the HAP transcription complex (Forsburg and Guarente, 1989; Gangloff *et al*., 1990; Rosenkrantz *et al*., 1994). Glutamate homeostasis, one of the central functions of *RTG*-dependent gene expression, exemplifies how the retrograde pathway serves to adapt cells to mitochondrial dysfunction. The levels of glutamate regulate the retrograde response via a negative feedback loop, whereby low glutamate levels activate and high glutamate levels repress *RTG*-dependent gene expression (Liu and Butow, 1999). Indeed, in cells with compromised mitochondrial function, mutations in any one of the *RTG* genes result in glutamate auxotrophy (Liao and Butow, 1993; Jia *et al*., 1997).

Although the exact mechanism of glutamate signaling is unclear, in respiratory-competent cells or in cells grown in the presence of high levels of glutamate in the medium, the basic helix–loop–helix/leucine zipper (bHLH/Zip) transcription factors, Rtg1p and Rtg3p, are present as an inactive complex in the cytoplasm (Sekito *et al*., 2000). In respiratory-deficient cells or in cells grown in medium lacking glutamate, these factors accumulate in the nucleus by processes requiring Rtg2p, a novel cytoplasmic protein with an N-terminal ATP-binding domain similar to the hsp70/actin/sugar kinase superfamily of ATP-binding proteins (Bork *et al*., 1992). Rtg2p thus acts as a proximal sensor of mitochondrial dysfunction, perhaps via glutamate levels, to regulate the subcellular localization of Rtg1p and Rtg3p (Sekito *et al*., 2000).

Recent studies have shown that the RTG pathway also responds to the quality of the nitrogen source in the medium and to the target of rapamycin (TOR) kinase pathway (Komeili *et al*., 2000; Shamji *et al*., 2000). In particular, when cells are grown in a poor nitrogen source, or when the TOR kinase pathway is inhibited by rapamycin, Rtg1p and Rtg3p translocate from the cytoplasm to the nucleus in an Rtg2p-dependent manner to activate target gene expression, similarly to the

Rtg2p-dependent nuclear accumulation of Rtg1p and Rtg3p in cells with dysfunctional mitochondria (Sekito *et al*., 2000). Together, these studies show that pathways of carbohydrate and nitrogen metabolism are linked together via *RTG*-dependent gene expression.

To identify additional regulatory genes in the RTG pathway, we searched for mutations that could bypass the requirement of *RTG2* for *CIT2* expression. This search led to the identification of *LST8*, an essential gene encoding a seven WD40-repeat protein that has been implicated in amino acid permease transport from the Golgi to the cell surface (Roberg *et al*., 1997a). We show that some, but not all, mutations in *LST8* can bypass the requirement of Rtg2p for Rtg1p/Rtg3p-dependent gene expression. We conclude that Lst8p functions as a negative regulator of *RTG*-dependent gene expression, acting at least at two sites, one upstream of Rtg2p at the level of external glutamate sensing and the other between Rtg2p and Rtg1p–Rtg3p.

Results

Isolation of RTG2 bypass mutants

To identify new genes in the retrograde pathway, we carried out a genetic screen to identify mutants that could bypass the requirement of Rtg2p for *CIT2* expression. Using an *rtg2Δ* strain with a *CIT2-lacZ* reporter gene integrated at the *URA3* locus, cells were mutagenized and screened for colonies that appeared blue on X-gal plates. This screen yielded 14 candidates that were selected as potential *rtg2Δ* bypass mutants, designated *rtb* (Rtg two bypass). Northern blot analysis revealed that all of the *rtb* isolates also had high levels of expression of the endogenous *CIT2* mRNA (data not shown), indicating that the *rtg2Δ* bypass phenotype was not peculiar to the reporter gene. Standard genetic tests showed that the *rtg2Δ* bypass phenotype of each isolate was recessive and due to a single gene mutation. Further, the isolates could be placed into two complementation groups, *rtb1* and *rtb2*. Five independent *rtb2* mutants were obtained, and the properties of some of them are described here; details of the *rtb1* complementation group will be described elsewhere.

Preliminary characterization of one of the *rtb2* isolates (*rtb2-5*) showed that the block in *CIT2-lacZ* expression due to the *rtg2Δ* mutation was completely reversed by the *rtb2-5* mutation (Figure 1A). Similar results were obtained for the other *rtb2* mutants. To exclude the possibility that the *rtb2* mutations activated an alternate pathway for *CIT2* expression, we introduced an *rtg3* null mutation into *rtg2Δ rtb2-5* mutant cells. *CIT2-lacZ* expression was abolished in the triple mutant (Figure 1A), suggesting that the *rtg2Δ* bypass does not involve the recruitment of alternative transcriptional activators.

RTB2 is LST8

To identify the *RTB2* gene, *rtg2Δ rtb2-5* mutant cells were transformed with a centromere-based wild-type yeast genomic library and screened for transformants that restored *CIT2-lacZ* expression to the low level observed in otherwise wild-type *rtg2Δ* cells. Complementing plasmids were obtained, and subsequent subcloning yielded a single gene, *LST8*, which was necessary and

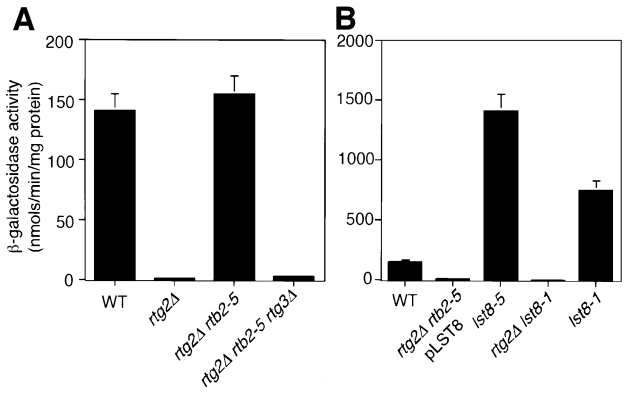

Fig. 1. *RTB2/LST8* is a negative regulator of *CIT2* expression. (**A**) *RTB2* regulates *CIT2* expression upstream of *RTG3*, and downstream of *RTG2*. Wild-type (PSY142), *rtg2Δ* (PSY142-rtg2), *rtg2Δ lst8-5* (RBY426) and *rtg2Δ rtb2-5 rtg3Δ* (RBY427) cells were grown in YPD medium to mid-log phase and collected for β-galactosidase activity analysis to determine transcriptional activation of an integrated *CIT2-lacZ* reporter gene. β-galactosidase assays on whole-cell extracts were carried out in triplicate as described in Materials and methods. (**B**) *RTB*2 is *LST8*. Wild-type PSY142 cells and *rtg2Δ lst8-5* transformed with pRS416-LST8, *lst8-5*, *rtg2Δ lst8-1* and *lst8-1* derivatives were grown in YNBcasD medium plus uracil (omitted in cells transformed with pRS416-LST8) to mid-log phase, and *CIT2-lacZ* reporter gene expression was determined by β-galactosidase activities.

sufficient to abolish reporter gene expression in each of the *rtg2Δ rtb2* isolates, as is shown for *rtg2Δ rtb2-5* cells (Figure 1B). To verify that *RTB2* and *LST8* are the same, we carried out complementation tests by examining *CIT2-lacZ* expression in diploids obtained from a cross between the *rtg2Δ rtb2-5* strain and an *rtg2Δ lst8Δ::LEU2* strain carrying a wild-type copy of *LST8* on a centromeric plasmid. The resulting diploids were cured of the plasmid and tested for *CIT2-lacZ* expression. We found that *CIT2-lacZ* expression was high in such diploids (data not shown), indicating that *RTB2* is *LST8*. Finally, as described in the next section, we identified point mutations in the *LST8* coding region for each of the *rtb2* mutant alleles identified in our screen. Hereafter, we will refer to *RTB2* as *LST8*. When the *lst8* mutant alleles were examined singly, as is shown for *lst8-5* (Figure 1B), *CIT2-lacZ* expression was nearly 10-fold higher than its expression in wild-type cells. Altogether, these data show that *LST8* is a negative regulator of *RTG* gene function.

The lst8-1 mutant allele does not bypass the rtg2Δ mutation

LST8 was shown previously to encode an essential seven WD-repeat protein that functions in the delivery of Gap1p, and possibly other amino acid permeases, to the cell surface (Roberg *et al*., 1997a). In that study, a mutant allele of *LST8* (*lst8-1*) was identified as a synthetic lethal with the *sec13-1* mutation. To determine whether the *lst8-1* mutation had similar effects on *RTG*-dependent gene activity, the *lst8-1* allele was transplaced into the *LST8* locus of wild-type and *rtg2Δ* cells and the resultant transformants assayed for *CIT2-lacZ* expression (Figure 1B). Surprisingly, unlike the *lst8* mutants we obtained, the *lst8-1* allele was unable to bypass the *rtg2Δ* mutation, although by itself it resulted in an ~4-fold activation of *CIT2-lacZ* expression. These findings raise

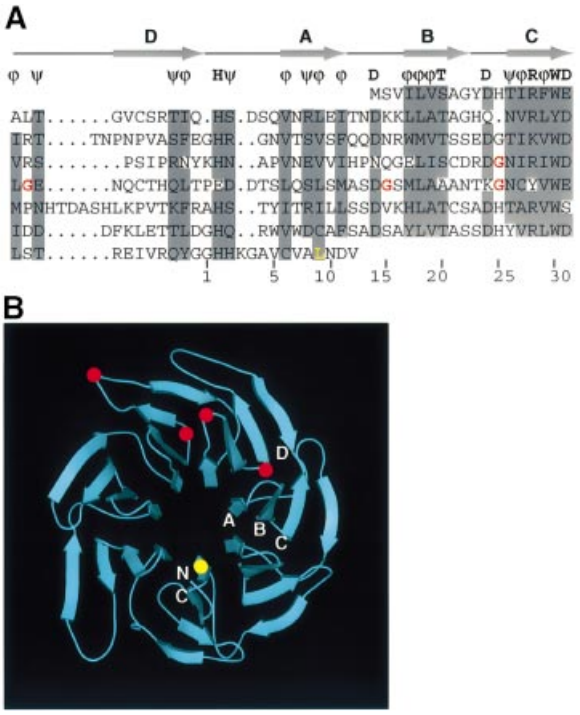

Fig. 2. Analysis of *lst8* mutations. (**A**) Alignment of the seven WD repeats in Lst8p. Five or more conserved residues among seven WD repeats are highlighted in gray, and the consensus sequence formed is shown above the alignment; φ designates any hydrophobic residue and ψ designates any hydrophilic residue; T designates serine or threonine. Based on the alignment between Lst8p and the G protein β1 subunit (ter Haar *et al*., 1998), each WD repeat is likely to consist of four β-strands, indicated by arrows A, B, C and D. The last row gives a canonical numbering system for residues of a WD repeat. The red type indicates mutations identified in our screen: *lst8-2*, Gly146 to glutamate; *lst8-3*, Gly138 to aspartate; *lst8-4* and *lst8-6*, Gly181 to aspartate; *lst8-5*, Gly171 to aspartate. The yellow type indicates the *lst8-1* mutation identified as a synthetic lethal with *sec13* (Roberg *et al*., 1997a). (**B**) Three-dimensional model of Lst8p. The model was created by Swiss-Model (Peitsch, 1996), an automated comparative protein modeling server, based on the homology between Lst8p and G protein β1 subunit. The image was prepared using programs Gl_render (http://www.hhmi.swmed.edu/external/Doc/G1_render.html), BOBSCRIPT (Esnouf, 1997), MOLSCRIPT (Kraulis, 1991) and Raster3D (Meritt, 1997). The positions of four β-strands A–D from WD repeat 6 are indicated. N and C designate the N- and C-terminal ends of Lst8p. Locations of the *lst8*-(*2–5*) mutations are indicated by the red dots, and the *lst8-1* mutation by the yellow dot.

the possibility that Lst8p has separable functions, a notion that will be developed later in this report.

Sequencing of the five *lst8* mutants we isolated showed that each contained a single missense mutation of a glycine residue, one in WD repeat 4 and the remainder in WD repeat 5 (Figure 2A). Two of the mutants contained the identical mutation at position 181 in WD repeat 5. Based on the design of our *rtg2Δ* bypass screen (see Materials and methods), those mutants were likely to have arisen independently. All of the mutated glycine residues were localized to tight turns in the protein. WD repeat 5 is the most divergent among the seven, lacking the conserved His2, Ser/Thr20 and Asp24, and containing a four-residue insertion in the linker region leading to WD repeat 6. We also determined the mutation in the *lst8-1* allele identified by Roberg *et al*. (1997a), and found that there was a leucine to serine change at position 300, a residue

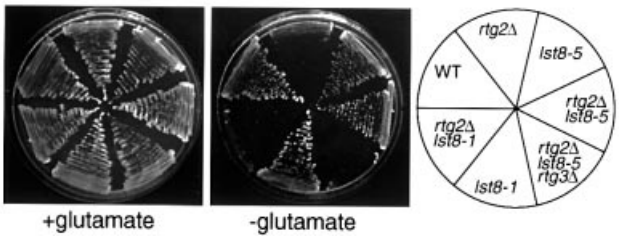

Fig. 3. The glutamate auxotrophy of an *rtg2Δ* mutation is rescued by the *lst8-5* but not the *lst8-1* mutation. Wild-type PSY142 cells, and *rtg2Δ*, *lst8-5*, *rtg2Δ lst8-5*, *rtg2Δ rtb2-5 rtg3Δ*, *lst8-1* and *rtg2Δ lst8-1* derivatives were streaked on YNBD medium supplemented or not with 0.02% glutamate as indicated, and with uracil, leucine and lysine, and incubated at 30°C for 2–3 days.

conserved among all seven WD repeats of Lst8p, as well as in the WD repeats of the G protein β-subunit (Sondek *et al*., 1996). We have designated the different *lst8* mutant alleles obtained in this study *lst8-2* through *lst8-5*, and collectively as *lst8*-(*2–5*). Structural modeling of Lst8p suggests that the *lst8*-(*2–5*) mutations are clustered and localized on the surface of the protein, whereas *lst8-1* would be at an internal position on the opposite side of the protein (Figure 2B).

lst8-5, but not lst8-1, rescues the glutamate auxotrophy of an rtg2Δ mutant

A hallmark of *rtg* mutant cells is that they are glutamate auxotrophs when their mitochondrial function is reduced or compromised (Liao and Butow, 1993; Liu and Butow, 1999). Given the striking difference in the ability of the *lst8*-(*2–5*) mutant alleles versus that of the *lst8-1* allele to suppress the block in *CIT2* expression in *rtg2Δ* cells, we asked whether there was also a difference among these *lst8* mutants in their ability to reverse the glutamate auxotrophy of an *rtg2Δ* strain grown in glucose medium. As shown for *lst8-5* (Figure 3), all of the *lst8*-(*2–5*) mutant alleles restored glutamate prototrophy to *rtg2Δ* cells, which also depended on the presence of *RTG3*. In contrast, the *lst8-1* mutation was unable to restore glutamate prototrophy to *rtg2Δ* cells, consistent with the inability of that *lst8* allele to bypass the loss of *CIT2* expression in *rtg2Δ* cells. In all cases when *CIT2* reporter gene expression was high, Rtg3p was localized in the nucleus (data not shown). These findings not only underscore the difference between the *lst8*-(*2–5*) and *lst8-1* mutants, but suggest that a site of negative regulation of *RTG*-dependent gene expression by Lst8p is between Rtg2p and the Rtg1p–Rtg3p transcription complex.

Both lst8-5 and lst8-1 mutant cells are largely insensitive to glutamate repression

To investigate further the regulation of *RTG*-dependent gene expression by Lst8p, we examined the effects of the addition of glutamate to the growth medium on *CIT2-lacZ* expression in *LST8*, *lst8-5* and *lst8-1* cells. In *LST8* cells grown in minimal medium, *CIT2-lacZ* expression was repressed >100-fold by the addition of 0.2% glutamate to the medium (Figure 4A). In *lst8-5* mutant cells, however, *CIT2-lacZ* expression was largely insensitive to glutamate repression, decreasing by only ~2-fold by the addition of 0.2% glutamate. This loss of sensitivity to glutamate repression was not the result of any bulk impairment of

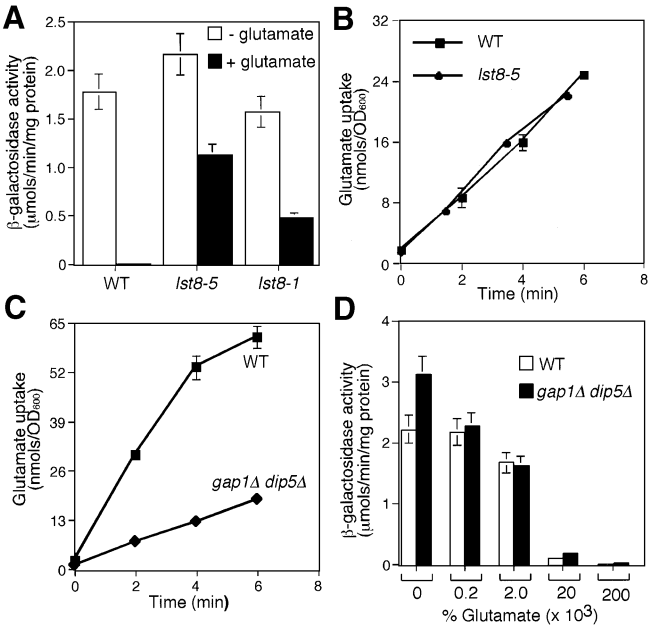

Fig. 4. The insensitivity of glutamate repression of *CIT2-lacZ* reporter gene expression in *lst8-5* mutant cells is not the result of a defect in glutamate uptake. (**A**) *Lst8-5* and *lst8-1* mutants are largely insensitive to glutamate repression of *CIT2-lacZ* reporter gene expression. Wild-type (PSY142), *lst8-1* and *lst8-5* mutant strains with an integrated copy of *CIT2-lacZ* reporter gene were grown in YNB5%D medium with or without 0.2% glutamate. Whole-cell extracts were prepared and β-galactosidase activities were determined as described in Materials and methods. (**B**) Glutamate uptake in wild-type and *lst8-5* mutant cells. Wild-type (PSY142) and *lst8-5* mutant strains were grown in YNBD medium and glutamate uptake was assayed as described in Materials and methods. Glutamate was present at a final concentration of 0.01%. (**C**) Glutamate uptake into wild-type and *gap1Δ dip5Δ* mutant strains. Wild-type (S288C) and a *gap1Δ dip5Δ* derivative strain were grown in YNBD medium, and the glutamate uptake assay was carried out as described in (B). (**D**) *CIT2-lacZ* reporter gene expression is still repressed by glutamate in *gap1Δ dip5Δ* mutant cells. Wild-type (S288C) and a *gap1Δ dip5Δ* mutant derivative strain were grown in YNB5%D medium with or without glutamate as indicated. Whole-cell extracts were prepared and β-galactosidase activities were determined as described in Materials and methods.

glutamate uptake, because *lst8-5* mutant cells showed the same kinetics of glutamate uptake as wild-type cells (Figure 4B). Interestingly, although the *lst8-1* mutation did not bypass the requirement for Rtg2p, it greatly reduced the glutamate repression of *CIT2-lacZ* expression.

We next asked whether glutamate would still repress *CIT2-lacZ* reporter gene expression in mutant cells that are defective in glutamate transport. In yeast, there are two major pathways for glutamate uptake, one involving the general amino acid permease encoded by *GAP1* and the other a specific glutamate/aspartate permease encoded by the *DIP5* gene (Regenberg *et al*., 1998). Accordingly, we examined the effects of inactivation of these genes on glutamate repression of *CIT2-lacZ* expression. In a *gap1Δ dip5Δ* double mutant, the rate of glutamate uptake was inhibited by ~85% (Figure 4C). Nevertheless, *CIT2-lacZ* expression in those double mutant cells was as sensitive to glutamate repression as in wild-type cells (Figure 4D). These data suggest that glutamate repression of *RTG*-dependent gene expression is not strictly dependent on glutamate uptake, and raise the possibility that glutamate repression may involve external glutamate sensing.

An Ssy1p–Ptr3p signal transduction cascade mediates glutamate repression of CIT2 expression

One candidate for an external glutamate sensor is Ssy1p, a plasma membrane protein with a predicted structure similar to that of amino acid permeases (Didion *et al*., 1998; Iraqui *et al*., 1999; Klasson *et al*., 1999). Previous studies have shown that amino acid induction of *AGP1*, which encodes a broad-specificity amino acid permease, as well as several other genes encoding amino acid permeases, was abolished in *ssy1* mutant cells (Didion *et al*., 1998; Iraqui *et al*., 1999). These amino acid inductive effects have been shown also to require Ptr3p, a peripheral plasma membrane protein, and it was proposed that Ptr3p interacts with Ssy1p as part of a signal transduction cascade for external amino acid sensing. In addition, induction probably requires Shr3p, a protein whose function is to facilitate the exit of Ssy1p from the endoplasmic reticulum (Klasson *et al*., 1999) and, hence, its targeting to the plasma membrane.

To investigate whether Ssy1p might mediate glutamate repression of *RTG* target gene expression, we first determined whether glutamate could still repress the activity of a *CIT2-lacZ* reporter gene in *ssy1Δ*, *ptr3Δ* and *shr3Δ* mutant cells. Figure 5A shows that the strong repression of *CIT2-lacZ* activity by glutamate in wild-type cells was essentially blocked in each of the mutant strains. The lack of significant glutamate repression of *CIT2-lacZ* expression in *ssy1Δ*, *ptr3Δ* and *shr3Δ* mutant cells is similar to that of the *lst8* mutants. However, unlike the *lst8-*(*2–5*) mutations, the loss of *CIT2* reporter gene expression in *rtg2Δ* cells was not reversed by the *ssy1Δ*, *ptr3Δ* or *shr3Δ* mutations (Figure 5A), and the double mutants *rtg2Δ ssy1Δ*, *rtg2Δ ptr3Δ* and *rtg2Δ shr3Δ* remained glutamate auxotrophs (Figure 5B). These data suggest that, like the *lst8-1* mutation, the *ssy1Δ*, *ptr3Δ* and *shr3Δ* mutations affect *RTG* gene function upstream of *RTG2*.

We next measured the rate of glutamate uptake in *ssy1Δ* cells, and found that glutamate uptake was inhibited by only ~50% (Figure 6A). These data are in agreement with a previous report showing a modest decrease in glutamate uptake in *ssy1* mutant cells (Klasson *et al*., 1999). To provide additional evidence that glutamate repression of *CIT2-lacZ* expression is not a function of glutamate uptake, we overexpressed Dip5p or Gap1p in *ssy1Δ* cells and determined the rate of glutamate uptake in those cells. Overexpression of these amino acid permeases in *ssy1Δ* cells restored rates of glutamate uptake to ~80% of the wild-type rate (Figure 6A), but *CIT2-lacZ* expression remained insensitive to glutamate repression (Figure 6B). Although the level of glutamate repression of *CIT2-lacZ* expression in wild-type cells overexpressing Dip5p and Gap1p was somewhat less than we observed in wild-type control cells, possibly because the high level of expression of these plasma membrane proteins competes for Ssy1p targeting, it is clear that in *ssy1Δ* cells overexpressing these amino acid permeases, glutamate completely fails to repress *CIT2-lacZ* reporter gene expression. Altogether, these data support the view that sensing via the Ssy1p–Ptr3p signal transduction pathway, rather than

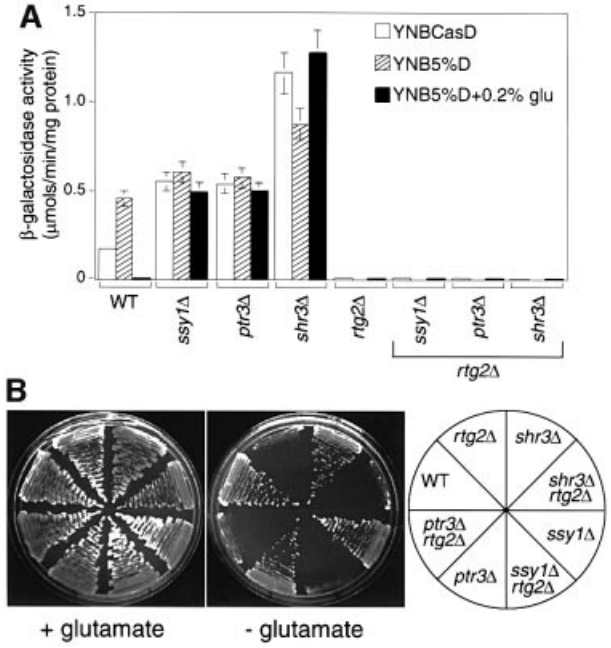

Fig. 5. Effects of *shr3Δ*, *ssy1Δ* and *ptr3Δ* mutations on glutamate repression, *rtg2Δ* suppression and glutamate auxotrophy. (**A**) *CIT2-lacZ* expression. Transformants of wild-type (PLY126), *shr3Δ* (PLY151-ura3), *ssy1Δ* (HKY20), *ptr3Δ* (HKY31), *rtg2Δ* (PLY126-rtg2), *shr3Δ rtg2Δ* (PLY151-rtg2), *ssy1Δ rtg2Δ* (HKY20-rtg2) and *ptr3Δ rtg2Δ* (HKY31-rtg2) strains containing a *CIT2-lacZ* reporter gene on the centromere-based plasmid pCIT2-lacZ were grown to mid-log phase in the media indicated in the figure, and β-galactosidase activities were determined in extracts as described in Materials and methods. Strains *rtg2Δ* (PLY126-rtg2), *shr3Δ rtg2Δ* (PLY151-rtg2), *ssy1Δ rtg2Δ* (HKY20-rtg2) and *ptr3Δ rtg2Δ* (HKY31-rtg2) were not tested for *CIT2-lacZ* expression when grown in YNB5%D medium because they are glutamate auxotrophs. (**B**) The *shr3Δ*, *ssy1Δ* and *ptr3Δ* mutations cannot rescue glutamate auxotrophy of *rtg2Δ* cells. Cells were streaked on YNBD medium with or without 0.02% glutamate and incubated at 30°C for 2–3 days.

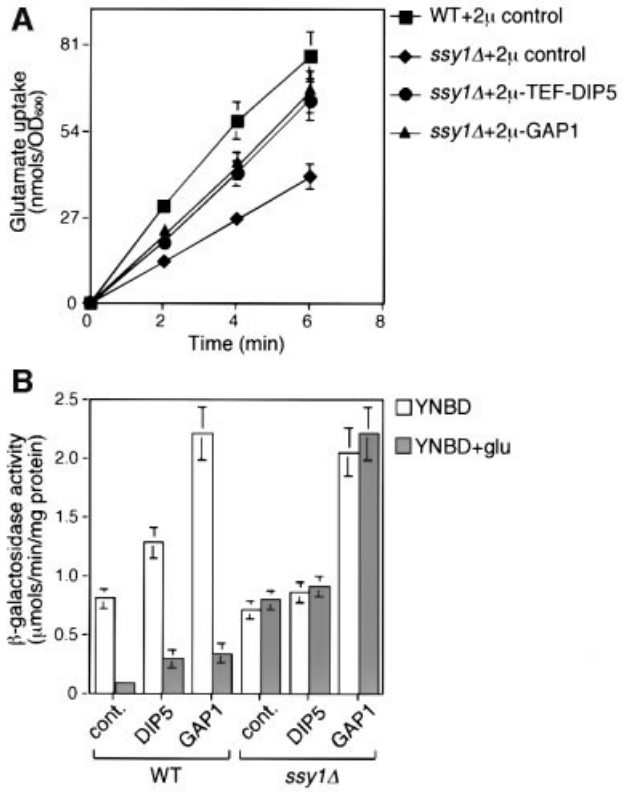

Fig. 6. The loss of glutamate repression of *CIT2-lacZ* reporter gene expression in *ssy1Δ* cells is uncoupled from glutamate uptake. (**A**) Glutamate uptake in *ssy1Δ* cells overexpressing Dip5 and Gap1. *ssy1Δ* (HKY20) cells were transformed with the 2μ control plasmid (empty vector), 2μ-TEF-DIP5 or 2μ-GAP1 plasmids, and the resultant transformants, including wild-type cells transformed with the 2μ control plasmid, were analyzed for glutamate uptake as described in Figure 4B. (**B**) Glutamate repression of *CIT2-lacZ* reporter gene expression in *ssy1Δ* cells overexpressing Dip5p and Gap1p. The various transformants described in (A) above were transformed with the pCIT2-lacZ reporter gene construct and the transformants were grown in YNBD medium with or without 0.2% glutamate. Whole-cell extracts were prepared and β-galactosidase activities were determined as described in Materials and methods.

glutamate uptake, is the major factor responsible for glutamate repression of *RTG*-dependent gene expression.

We next determined the amino acid specificity for repression of *CIT2* reporter gene expression in wild-type cells. *CIT2-lacZ* expression was repressed most strongly in cells grown in the presence of glutamate or glutamine, whereas its expression was reduced <3-fold in the presence of other amino acids (Figure 7). In an *ssy1Δ* mutant, repression of *CIT2-lacZ* expression by glutamate and glutamine was eliminated and, in most instances, the modest repression by the other amino acids was also eliminated. These data suggest that specific and non-specific repression of *RTG* target gene expression is effected through Ssy1p.

Genome-wide transcription analysis of genes regulated by glutamate

Because glutamate can act as both a repressor and an activator of gene expression (Iraqui *et al*., 1999), with both effects operating through the Ssy1p–Ptr3p signal transduction pathway, we wished to obtain a more global view of glutamate-regulated genes. For this purpose, we carried out microarray analysis of ~6200 yeast genes to determine how their relative mRNA abundance is affected by the addition of glutamate to the medium. A number of studies have shown that different quality nitrogen sources give rise to different patterns of gene expression (Cardenas *et al*., 1999; Hardwick *et al*., 1999; Komeili *et al*., 2000). Assessment of the quality of different nitrogen sources has been complicated by the fact that there is variation among strains in their response to the utilization of different nitrogen sources, including ammonia and glutamate. Accordingly, as we have done for all of the experiments described here on the effects of glutamate on *RTG*-dependent gene expression, glutamate was added to medium already containing ammonia, so as to avoid comparing different, sole nitrogen sources.

Strain S288C was grown to mid-log phase in YNB5%D medium with or without supplementation with 0.2% glutamate. mRNA was isolated, and labeled cDNAs were prepared by reverse transcription in the presence of Cy3- or Cy5-labeled nucleotides. These labeled cDNAs were then mixed and hybridized in duplicate with both dye configurations to yeast whole-genome arrays. Applying a stringent cut-off of an average of a 2.5-fold difference for

individual genes, we found 45 genes whose expression was affected by glutamate (Table I); of these, 31 genes were repressed and 14 were induced. Among the repressed genes, 12 are known to be subject to nitrogen catabolite repression (NCR), including *MEP2*, encoding an ammonia permease, and *GAP1*, encoding a general amino acid permease. The repression of these NCR genes by glutamate suggests that ammonia is not a preferred nitrogen source for strain S288C, and that the repression of the NCR genes by glutamate is likely to be due to the combined effect of glutamate and ammonia to generate glutamine, which is a preferred nitrogen source. As expected, three *RTG* target genes, *ACO1*, *CIT2* and *IDH2*, were among the glutamate-repressible genes.

Among 14 genes induced by glutamate, six encode amino acid permeases, five of which, *BAP2*, *BAP3*, *AGP1*, *GNP1* and *VAP1/TAT1*, belong to the Cluster I amino acid permease family that shows a broad substrate specificity (Regenberg *et al*., 1999). Induction of these genes by external amino acids is Ssy1p dependent (Iraqui *et al*., 1999), underscoring the conclusion that glutamate can effect both induction and repression of gene expression.

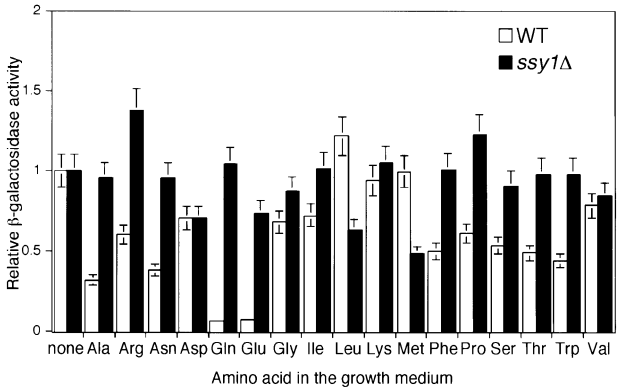

Fig. 7. Effects of different amino acids on *CIT2-lacZ* reporter gene expression in wild-type and *ssy1Δ* cells. Wild-type (PLY126) and *ssy1Δ* (HKY20) strains transformed with a centromere-based plasmid pCIT2-lacZ were grown to mid-log phase in YNB5%D medium supplemented with 0.1% of the indicated amino acids. Whole-cell extracts were prepared and β-galactosidase activities were determined as described in Materials and methods.

Discussion

To identify additional components of the *RTG* pathway, we sought mutants that could bypass the dependence on Rtg2p, but still require the Rtg1p–Rtg3p transcriptional activators for target gene expression. The current work shows that certain mutations in the seven WD40-repeat protein, Lst8p, can bypass the requirement for Rtg2p, and establishes that in wild-type cells, Lst8p is a negative regulator of *RTG*-dependent gene expression. As diagrammed in the model of Figure 8, we suggest that Lst8p can regulate *RTG*-dependent gene expression at two sites, one downstream of Rtg2p and the other upstream at the level of external glutamate repression of the RTG pathway.

Because in both *lst8-1* and *lst8-(2–5)* mutant cells, expression of the *CIT2-lacZ* reporter gene was constitutive and insensitive to repression by glutamate, one simple way that Lst8p could regulate the *RTG* pathway is by controlling the cells' ability to take up glutamate. Indeed, a previous study demonstrated that the general amino acid permease, Gap1p, failed to be targeted to the plasma membrane in *lst8-1* mutant cells (Roberg *et al*., 1997a). Our results suggest, however, that glutamate repression of *RTG*-dependent gene expression is more likely to occur through glutamate sensing involving the Ssy1p–Ptr3p signal transduction pathway rather than by processes dependent on glutamate uptake. First, inactivation of the Ssy1p–Ptr3p pathway eliminated glutamate repression of *RTG*-dependent gene expression, but glutamate uptake in *ssy1Δ* cells was inhibited only by ~50%; the latter result is in agreement with a previous study (Klasson *et al*., 1999). Secondly, glutamate repression of the *RTG* pathway was largely unaffected by inactivation of both the general (Gap1) and glutamate/aspartate-specific (Dip5p) amino acid permeases. Finally, overexpression of Gap1p and Dip5p in *ssy1Δ* cells restored the rate of glutamate uptake to ~80% of that of wild-type cells, yet *CIT2* expression, as in *ssy1Δ* cells alone, was insensitive to glutamate repression. These data suggest that, through Ptr3p-coupled glutamate signaling, the binding of glutamate to Ssy1p would send a signal (inhibitory) to the *RTG* pathway, as has been proposed for the effects of other amino acids in the Ssy1p/Ptr3p-dependent activation of gene expression in the amino acid inductive pathway (Didion *et al*., 1998; Klasson *et al*., 1999; Iraqui *et al*., 1999). Despite the loss of glutamate repression of the *RTG* pathway in *ssy1Δ*, *ptr3Δ*, *shr3Δ* as well as in *lst8-1* cells, none of these mutations can bypass the requirement of Rtg2p for *CIT2* expression as can the *lst8(2–5)* mutations.

In considering the effect of glutamate on *RTG*-dependent gene expression, it is important to distinguish between the sensing of extra- and intracellular glutamate. In cells with compromised mitochondrial function, intracellular glutamate supplies are maintained by *RTG* regulation of expression of the four TCA cycle genes responsible for the synthesis of α-ketoglutarate (Liu and Butow, 1999), by increased expression of genes encoding enzymes that function in anaplerotic pathways, small molecule transport and peroxisomal activities that converge to supply acetyl-CoA, oxaloacetate and citrate to the TCA cycle, and by increased expression of genes that enable cells to utilize poor nitrogen sources (Epstein *et al*., 2001b). In contrast, when glutamate is available in the medium, the *RTG*-dependent gene expression is down-regulated by the *SSY1–PTR3* signal transduction pathway.

Although the *RTG* pathway is also insensitive to glutamate repression in *lst8-(2–5)* mutant cells, it is quite possible that the Lst8p regulatory site(s) downstream of Rtg2p may have no direct connection to glutamate regulation of the pathway. The WD40-repeat motif was identified originally in the β-subunit of heterotrimeric G proteins (Fong *et al*., 1986) and subsequently has been found in a wide spectrum of regulatory proteins, where it functions in mediating protein–protein interactions. Thus Lst8p could be a component of a complex that tethers Rtg1p–Rtg3p in the cytoplasm. In this model, the loss of glutamate repression of *RTG*-dependent gene expression in *lst8-(2–5)* mutant cells would be a consequence of the constitutive localization of the Rtg1p–Rtg3p complex in

Table I. Genome-wide effects of glutamate on gene expression

Gene expression	ORF	Gene	Gene product/function	Fold change
Repressed				
NCR target genes				
	YNL142W	*MEP2*	ammonia permease of low capacity and high affinity	14.3
	YKR039W	*GAP1*	general amino acid permease	14.0
	YKR034W	*DAL80*	negative regulator of multiple nitrogen catabolic genes	12.4
	YLR158C	*ASP3C*	L-asparaginase II, extracellular	9.0
	YIR032C	*DAL3*	ureidoglycolate hydrolase	7.7
	YLR155C	*ASP3A*	L-asparaginase II, extracellular	7.5
	YLR157C	*ASP3B*	L-asparaginase II, extracellular	7.4
	YLR160C	*ASP3D*	L-asparaginase II, extracellular	5.8
	YJR152W	*DAL5*	allantoate permease	5.1
	YIR031C	*DAL7*	malate synthase 2	4.5
	YLR142W	*PUT1*	proline oxidase	3.3
	YOR348C	*PUT4*	putative proline-specific permease	2.7
RTG target genes				
	YLR304C	*ACO1*	aconitase, mitochondrial	6.5
	YCR005C	*CIT2*	non-mitochondrial citrate synthase	3.3
	YOR136W	*IDH2*	NAD^+-dependent isocitrate dehydrogenase	3.1
Others				
	YKR033C		protein of unknown function	11.4
	YLR348C	*DIC1*	mitochondrial dicarboxylate transport protein	5.4
	YLR349W		protein of unknown function	4.4
	YBR182C	*SMP1*	probable transcription factor	4.3
	YBR294W	*SUL1*	probable sulfate transport protein	4.2
	YPL058C	*PDR12*	multidrug resistance transporter	3.6
	YNL208W		protein of unknown function	3.2
	YOR135C		protein of unknown function	2.9
	YJL172W	*CPS1*	carboxypeptidase yscS	2.9
	YGL045W		protein of unknown function	2.7
	YML131W		putative NAD-dependent oxidoreductase	2.7
	YLR161W		protein of unknown function	2.7
	YER124C		protein of unknown function	2.6
	YLL053C		aquaporin water channel protein	2.6
	YIL121W		member of MFS-MDR family of multidrug permeases	2.5
	YGL028C	*SCW11*	soluble cell wall protein	2.5
Induced				
Amino acid permeases				
	YDR046C	*BAP3*	valine transporter	22.9
	YDR068	*BAP2*	probable amino acid permease for leucine, isoleucine and valine	
	YDR			20.0
	YCL025C	*AGP1*	amino acid permease	12.1
	YBR069C	*VAP1*	probable amino acid transport protein	12.0
	YDR508C	*GNP1*	high-affinity glutamine permease	6.1
	YGR055W	*MUP1*	high-affinity methionine permease	4.3
Proteases				
	YIR039C	*YPS6*	GPI-anchored aspartic protease	6.6
	YOL154W		protein with similarity to zinc metalloproteinases	2.8
Other				
	YDR509W		protein of unknown function	4.8
	YNL160W	*YGP1*	glycoprotein synthesized in response to nutrient limitation	3.8
	YMR011W	*HXT2*	high-affinity hexose transporter-2	3.6
	YBR092C	*PHO3*	acid phosphatase, constitutive	3.2
	YOL158C	*ENB1*	enterobactin transporter	3.0
	YNL065W		member of MFS-MDR family of multidrug permeases	2.7

Four independent cultures of strain S288C were grown to mid-log phase in YNB5%D with or without 0.2% glutamate. After isolation of $poly(A)^+$ RNA from each, labeled cDNAs were made with either Cy3 or Cy5 and mixed with the oppositely labeled cDNA from treated or untreated samples. The indicated fold changes in gene expression were determined as described in Epstein *et al*. (2001a) using a cut-off of 2.5-fold change in the average of the expression ratios from two independent RNA preparations, each repeated with reversal of Cy3 and Cy5 labeling.

the nucleus. An alternative model is that Lst8p could bind to and regulate the activity of a kinase or phosphatase, thus effecting a change in the phosphorylation state of Rtg3p; this, in turn, would dictate Rtg3p's nuclear entry or exit. It remains to be determined how internal glutamate is sensed by the *RTG* pathway and how Rtg2p functions to transduce glutamate signals to the Rtg1p–Rtg3p transcription complex.

What could be the molecular basis for the proposed dual sites of action of Lst8p as diagrammed in the model of Figure 8? We suggest that there are at least two domains of Lst8p, one functioning in the assembly or activity of the Ssy1p–Ptr3p signal transduction complex and the other in the negative regulation of *RTG*-dependent gene expression downstream of Rtg2p. In addition to the genetic evidence already summarized, clearly defining sites of Lst8p action

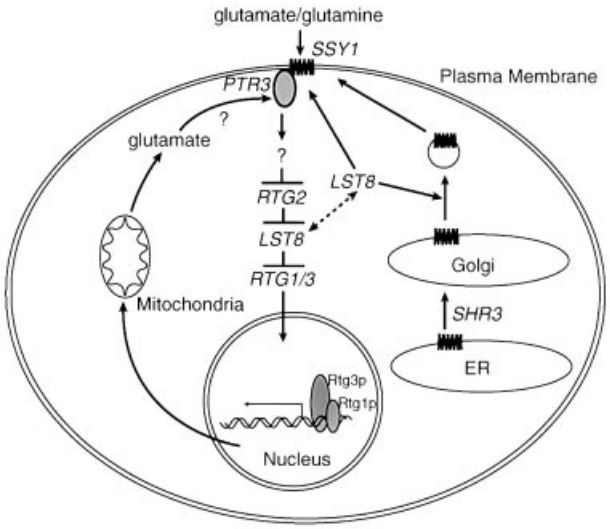

Fig. 8. Model for dual regulation of *RTG* gene functions by Lst8p. Once on the cell surface, Ssy1p binds to glutamate or glutamine and sends an inhibitory signal to Rtg2p through Ptr3p. As revealed by the *lst8-1* mutation, Lst8p might affect targeting or assembly of the Ssy1p–Ptr3p system, or its signal transduction function as suggested by Roberg *et al*. (1997a), and thus act upstream of Rtg2p. Lst8p is also proposed to function downstream of Rtg2p, as revealed by the *lst8-(2–5)* mutations. Intracellular glutamate is hypothesized to be sensed by Ptr3p (Klasson *et al*., 1999), or some additional component of the Ssy1p signaling pathway. Repressive signals generated by glutamate would lock Lst8p in its '*RTG* inhibition' state so that less Lst8p would be available for its function in Ssy1p–Ptr3 signaling; this would have the effect of attenuating glutamate repression of *RTG* gene functions.

both upstream and downstream of Rtg2p, it is striking that the *lst8-(2–5)* mutations are clustered and localize to a region of Lst8p that is predicted to be on a side of the protein opposite to that of the *lst8-1* mutation (Figure 2B). WD40-repeat proteins usually adopt a β-propeller structure, which can use one or two blades to interact with other proteins without affecting the other blades (Goodman *et al*., 1997; ter Haar *et al*., 1998). We propose that the distinctive phenotypes of two types of *lst8* mutations are due to defects in the interaction of the protein with different factors. The dual functionality of Lst8p may facilitate glutamate homeostasis by participating in a negative feedback loop for regulating glutamate levels. In the presence of high concentrations of external glutamate, signals generated by Ssy1p could 'lock' Lst8p in a form that functions to sequester the Rtg1p–Rtg3p complex in the cytoplasm. Thus, less Lst8p would be available for the Ssy1p signal transduction system, where it plausibly might function in targeting or post-translational modification of one or more components of that system. This model could explain the observation that glutamate blocks the targeting of Gap1p to the plasma membrane (Roberg *et al*., 1997a,b), and further suggests a means for attenuating glutamate repression as the concentration of glutamate in the medium decreases. Consistent with this 'switching' model, we have observed that in *rtg* mutant cells grown in rich medium, there is an ~10-fold induction of *AGP1* expression (data not shown). This finding could indicate that Lst8p has been released from its site(s) downstream of Rtg2p so that more of the protein is available for the *SSY1–PTR3* pathway. Altogether, these findings imply that Lst8p might be localized to more than one cellular compartment. Although the specific intracellular location of Lst8p has not been established, preliminary cell fraction studies suggest that Lst8p may indeed be localized to different cellular compartments (unpublished observations). Further studies will be required to clarify these issues.

Ssy1p has been shown to be involved in the amino acid inductive expression of some amino acid permeases (Didion *et al*., 1998; Iraqui *et al*., 1999), a peptide transporter, Ptr2p (Didion *et al*., 1998), and, as shown here, in glutamate repression. These findings prompted us to carry out a genome-wide transcriptional analysis on the effects of glutamate on gene expression. Microarray experiments revealed three major pathways that were affected by glutamate: NCR; the *RTG* pathway; and the amino acid inductive pathway. Interestingly, Ssy1p appears to be involved in all three of these pathways (Didion *et al*., 1998; Iraqui *et al*., 1999; Klasson *et al*., 1999). One set of genes whose expression was affected by glutamate in our microarray analysis was amino acid-inducible genes, *BAP2*, *BAP3*, *GNP1*, *VAP1/TAT1* and *AGP1*. Their transcription has been shown to be dependent on Ssy1p (Iraqui *et al*., 1999). Although, compared with hydrophobic amino acids, glutamate and glutamine are strong repressors of *RTG* gene function, they are weak inducers for *AGP1* expression. We consistently observed that the addition of 0.01% glutamate to the growth medium resulted in a 4- to 5-fold greater induction of *AGP1* compared with addition of 0.2% glutamate (data not shown), further supporting the Lst8p switching model discussed above. The amino acid induction of *PTR2* transcription involves a transcriptional repressor Cup9p and a N-end rule pathway component, Ubr1p (Goodman *et al*., 1997; Byrd *et al*., 1998). Is there any cross-regulation among these pathways? We found in preliminary experiments (data not shown) that *ubr1*, *ure2* or *gln3* deletion mutations have no significant effect on the expression of the *RTG* target gene, *CIT2*, and, conversely, that *rtg* mutations do not affect expression of the NCR target gene, *GLN1*. These findings suggest that there is little or no direct cross-regulation of these pathways downstream of Ssy1p–Ptr3p.

Recent work has shown that the TOR (target of rapamycin) kinase signaling pathway, which is involved in nutrient sensing, also affects *RTG*-dependent gene expression in the same manner as the retrograde pathway, namely by affecting the subcellular localization of Rtg1p and Rtg3p in an Rtg2p-dependent manner (Komeili *et al*., 2000). Inhibition of the TOR kinase pathway by treating cells with rapamycin or by growing them in a poor nitrogen source promotes the dissociation of Ure2p from a cytoplasmic, phosphorylated form of a positive regulator of the NCR pathway, Gln3p, resulting in Gln3's dephosphorylation and nuclear accumulation (Beck and Hall, 1999; Cardenas *et al*., 1999; Hardwick *et al*., 1999). Dephosphorylation of Gln3p is believed to be controlled by the type 2A-related phosphatase, Sit4p, and its inhibitor, Tap42p, an effector protein of the TOR pathway (Beck and Hall, 1999). An *sit4* deletion mutation, however, does not affect *CIT2* expression (unpublished data), suggesting that different TOR effectors may serve to regulate the NCR and *RTG* pathways.

Table II. *Saccharomyces cerevisiae* strains used

Strain	Genotype	Source
PSY142	*MATα ura3-52 leu2 lys2 ura3::CIT2-lacZ*	R.Butow
PSY142-rtg2	*MATα ura3-52 leu2 lys2 ura3::CIT2-lacZ rtg2Δ::ura3*	R.Butow
RBY346	*MATα ura3-52 leu2::LEU2-RTG2 lys2 ura3::CIT2-lacZ rtgΔ::ura3 lst8-5*	this study
RBY426	*MATα ura3-52 leu2 lys2 ura3::CIT2-lacZ rtg2Δ::ura3 lst8-5*	this study
RBY427	*MATα ura3-52 leu2 lys2 ura3::CIT2-lacZ rtg2Δ::ura3 lst8-5 rtg3Δ::LEU2*	this study
RBY254	*MATα ura3-52 leu2 lys2 ura3::CIT2-lacZ lst8-1*	this study
RBY264	*MATα ura3-52 leu2 lys2 ura3::CIT2-lacZ lst8-1 rtg2Δ::kanMX4*	this study
RBY418	*MAT***a** *ura3 leu2 lys2*	this study
RBY428	*MAT***a**/α *ura3/ura3 leu2/leu2 lys2/lys2 LST8/lst8Δ::LEU2* [pLST8]	this study
RBY419	*MAT***a** *ura3 leu2 lys2 rtg2Δ::kanMX4*	this study
RBY420	*MAT***a** *ura3 leu2 lys2 lst8Δ::LEU2 pLST8*	this study
RBY421	*MAT***a** *ura3 leu2 lys2 lst8Δ::LEU2 rtg2Δ::kanMX4 pLST8*	this study
RBY422	*MAT***a**/α *ura3/ura3::CIT2-lacZ leu2/leu2 lys2/lys2 RTG2/rtg2Δ::ura3 LST8/lst8-5*	this study
RBY423	*MAT***a**/α *ura3/ura3::CIT2-lacZ leu2/leu2 lys2/lys2 rtg2Δ::ura3/rtg2Δ::kanMX4 LST8/lst8-5*	this study
RBY424	*MAT***a**/α *ura3/ura3::CIT2-lacZ leu2/leu2 lys2/lys2 rtg2Δ::ura3/rtg2Δ::kanMX4 lst8Δ::LEU2/lst8-5*	this study
ECY408	*MAT***a** *ura3 lst8-1*	C.Kaiser
S288C	*MAT***a** *ura3-52*	
M4276	*MAT***a** *ura3 gap1Δ dip5Δ::kanMX*	M.Kielland-Brandt
PLY126	*MAT***a** *ura3-52 lys2Δ201*	P.Ljungdahl
PLY151-ura3	*MAT***a** *ura3-52 lys2Δ201 ade2 shr3Δ1::ura3*	P.Ljungdahl
HKY20	*MAT***a** *ura3-52 lys2Δ201ssy1Δ13*	P.Ljungdahl
HKY31	*MAT***a** *ura3-52 lys2Δ201 ptr3Δ15*	P.Ljungdahl
PLY126-rtg2	*MAT***a** *ura3-52 lys2Δ201 rtg2::kanMX4*	this study
PLY151-rtg2	*MAT***a** *ura3-52 lys2Δ201 alde2 shr3Δ1::ura3 rtg2::kanMX4*	this study
HKY20-rtg2	*MAT***a** *ura3-52 lys2Δ201 ssy1Δ13 rtg2::kanMX4*	this study
HKY31-rtg2	*MAT***a** *ura3-52 lys2Δ201 ptr3Δ15 rtg2::kanMX4*	this study

Materials and methods

Strains

Strains used in this study are listed in Table II.

Growth media, growth conditions

Yeast strains were grown at 30°C in YPD (1% yeast extract, 2% bactopeptone and 2% dextrose), YNBcasD (0.67% yeast nitrogen base, 1% casamino acids and 2% dextrose) or minimal YNB medium (0.67% yeast nitrogen base) supplemented with 2 or 5% glucose (YNBD or YNB5%D, respectively), with or without Na glutamate (as indicated in the text and figures), and supplemented with the required nutrients.

Strain constructions

Standard genetic manipulations were carried out as described in Rose *et al*. (1990). RBY426 was isolated from the *rtg2* bypass screen described below. RBY426 was transformed with a *rtg3Δ::LEU2* disruption cassette to form RBY427. RBY254 was an *lst8-1* mutant obtained through a two-step gene replacement. RBY418 was derived from PSY142 lacking the integrated *CIT2-lacZ* reporter gene by mating-type switching of PSY142 cells transformed with the plasmid pGAL-HO (Herskowitz and Jensen, 1991). RBY418 was transformed with an *rtg2::kanMX4* disruption cassette to form RBY419. RBY420 is a meiotic segregant obtained following sporulation of the diploid RBY428 transformed with pRS416-LST8. RBY421 was constructed from RBY420 by transforming cells with pUC18-rtg2::kanMX4 digested with *Pst*I. Matings were carried out between RBY426 and RBY418, RBY419 or RBY421 to form RBY422, RBY423 or RBY424, respectively. *RTG2* disruptions in the strains PLY126-rtg2, PLY151-rtg2, HKY20-rtg2 and HKY31-rtg2 were carried out by transformation of the *rtg2::kanMX4* disruption cassette into their respective parental strains.

Plasmid constructs

Construction of *rtg3Δ::LEU2* disruption cassette was described previously (Liu and Butow, 1999). To construct a *rtg2Δ::kanMX4* cassette derivative, the primer set 5′-AGGCACACTCTTCTTCAC-3′ and 5′-gactaagcttTGAACAACAAGAAGGTGCCC-3′ was used to amplify *RTG2*. After digestion with *Bgl*II and *Hin*dIII, *RTG2* DNA was ligated into the *Bam*HI and *Hin*dIII sites of pRS416 to form pRS416-RTG2. A 643 bp *Nde*I fragment of pRS416-RTG2 was cloned into the *Nde*I site of pUC18 to form pUC18-RTG2. A *kanMX4* disruption cassette module was inserted into the *Spe*I site to form pUC18-rtg2::kanMX4. The primer set 5′-gactactagtACCACCAGGACCAAAGCTTG-3′ and 5′-gactgtcgacCAAAAGCGGGAAGAAGTCA-3′ was used to amplify the 2.1 kbp *LST8* sequence. ECY408 genomic DNA was used to amplify the *lst8-1* mutant allele. The amplified DNA was digested with *Spe*I and *Sal*I and ligated into Yip352 or pRs416 to form, respectively, Yip352-lst8-1 and pRS416-lst8-1. Similarly, *LST8* was amplified from the PSY142 strain and isogenic *rtb2* mutant strains to construct pRS416-LST8, pRS416-lst8-2–6, respectively. pCIT2-lacZ was described previously (Liao *et al*., 1991; Liu and Butow, 1999). To obtain plasmid 2μ-LYS2, the pRS426 plasmid was digested with *Eco*RV and *Stu*I to remove part of the *URA3* sequence and replaced with a 5.2 kbp *LYS2* sequence from *Sma*I- and *Pst*I-digested YDp-K (Berben *et al*., 1991). To construct plasmid 2μ-TEF-DIP5, the primer pair 5′-gtcaACTAGTCTCTAAGTAATGAA-GATGCC-3′ and 5′-gtcaGTCGACGTGATACCTGTACACTATGG-3′ was used to amplify the *DIP5* open reading frame and 413 bp of 3′-untranslated region. The resultant PCR product was digested with *Spe*I and *Sal*I and ligated into the pRS426-TEF plasmid (Mumberg *et al*., 1995). The *URA3* sequence in the pRS426-TEF-DIP5 was replaced with *LYS2*, similarly to the construction of 2μ-LYS2. To construct 2μ-GAP1 plasmid, a 3.5 kbp *GAP1* sequence was obtained from *Spe*I- and *Sal*I-digested pPL247 (Ljungdahl *et al*., 1992) and ligated into pRS426. The resultant pRS426-GAP1 was digested with *Nco*I and *Stu*I to remove part of the *URA3* sequence and replaced with the same *LYS2* sequence described above.

Rtg2 bypass mutant screen

Ethyl methanesulfonate mutagenesis was carried out essentially as described previously (Liao and Butow, 1993). Cells were plated on YNBD plus X-gal and amino acid supplements. Fourteen *rtb* mutants were obtained that could be grouped into two complementation groups, *rtb1* and *rtb2*, as described in the text. The *rtb* mutants arose at a frequency of ~1 in 10^6 cells.

Isolation of the RTB2 (LST8) gene

A *URA3* centromeric *CIT2-lacZ* reporter gene was transformed into a RBY285 (*rtg2Δ rtb2-5*) derivative lacking the integrated version of the reporter gene. To isolate the *RTB2* gene, a wild-type genomic DNA library constructed in the centromeric vector p366 carrying a *LEU2* marker was screened for transformants of RBY285 that would abolish *CIT2-lacZ* expression. Two out of 8000 $LEU2^+$ transformants were obtained that had overlapping restriction maps. Subcloning in the centromeric plasmid pRS415 and retransformation revealed that a fragment containing the *LST8* gene was necessary and sufficient to complement the *rtb2-5* mutant phenotype.

β-galactosidase assays

β-galactosidase assays were carried out as previously described (Liu and Butow, 1999). For each plasmid–strain combination, assays were conducted in triplicate and independent experiments were carried out two or three times.

Assays for amino acid uptake

Amino acid uptake was assayed as described by Roberg *et al*. (1997a) except that for glutamate uptake, equal volumes of 0.2 mM ^{14}C-labeled glutamate and 59.1 mM unlabeled glutamate were mixed and diluted 50-fold into the assay mixture. At 2 min intervals, two samples of 100 µl each were diluted into 3 ml of ice-cold 5.9 mM glutamate to quench the reaction, and cells were collected by filtration and washed with 3× 5 ml of ice-cold water. L-[U-^{14}C]glutamic acid (250 mCi/mmol) was obtained from Amersham Pharmacia (Buckinghamshire, UK).

Microarray analysis

A fresh culture of S288C was diluted into 250 ml of YNB5%D with or without 0.2% glutamate. After overnight growth to OD_{600} 0.8, cells were collected and mRNA samples were prepared as described (Epstein *et al*., 2001b). Cy3- and Cy5-labeled cDNAs were prepared and hybridized to a microarray of 6219 yeast genes. Replica experiments were carried out using independent liquid cultures and with the opposite configuration of Cy3 and Cy5. The data were analyzed as described (Epstein *et al*., 2001a,b) using a cut-off of value of an average of 2.5-fold change in replica hybridizations.

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