Retrograde (RTG) signaling senses mitochondrial dysfunction and initiates readjustments of carbohydrate and nitrogen metabolism through nuclear accumulation of the heterodimeric transcription factors, Rtg1/3p. The RTG pathway is also linked to target of rapamycin (TOR) signaling, among whose activities is transcriptional control of nitrogen catabolite repression (NCR)-sensitive genes. To investigate the connections between these two signaling pathways, we have analyzed rapamycin sensitivity of the expression of the RTG target gene CIT2 and of two NCR-sensitive genes, GLN1 and DAL5, in respiratory-competent (p+) and -incompetent (p-) yeast cells. Here we have presented evidence that retrograde gene expression is separable from TOR regulation of RTG- and NCR-responsive genes. We showed that expression of these two classes of genes is differentially regulated by glutamate starvation whether in response to mitochondrial dysfunction or induced by rapamycin treatment, as well by glutamine or histidine starvation. We also showed that Lst8p, a component of the TOR1/2 complexes and a negative regulator of the RTG pathway, has multiple roles in the regulation of RTG- and NCR-sensitive genes. Lst8p negatively regulates CIT2 and GLN1 expression, whereas DAL5 expression is independent of Lst8p function. DAL5 expression depends on the GATA transcription factors Gln3p and Gat1p. Gat1p is translocated to the nucleus only upon TOR inhibition by rapamycin. Altogether, these data show that Rtg1/3p, Gln3p, and Gat1p can be differentially regulated through different nutrient-sensing pathways, such as TOR and retrograde signaling, and by multiple factors, such as Lst8p, which is suggested to have a role in connecting the RTG and TOR pathways.

The survival of cells depends on their ability to cope with environmental changes and with alterations in cellular functions through the deployment of adaptive responses in metabolic and regulatory networks. Mitochondria play an important role in cellular adaptation via a mitochondria-to-nucleus signaling pathway called retrograde regulation (1). In the budding yeast Sacccharomyces cerevisae, loss of respiratory function, for instance, in cells that lack mitochondrial DNA (p0 petites), leads to changes in the expression of a subset of nuclear genes that enable those cells to adapt to their respiratory-deficient state (2). In particular, a variety of anaplerotic pathways are activated in respiration-deficient cells that provide key metabolites such as acetyl-CoA and oxaloacetate to mitochondria to compensate for the block of the Krebs cycle. This allows respiratory-deficient cells to maintain the synthesis of α-ketoglutarate, a precursor to glutamate and glutamine, thus preventing amino acid starvation. The glyoxylate cycle gene, CIT2, encoding an isomerase of citrate synthase, and DLD3, encoding a cytoplasmic lactate dehydrogenase, are among the genes activated in respiratory-deficient cells (3, 4). Their expression is positively regulated by the heterodimeric transcription factors Rtg1p and Rtg3p and Rtg2p, a novel cytoplasmic protein with an N-terminal ATP binding domain that is required for the relocation of Rtg1/3p from the cytoplasm to the nucleus when the retrograde pathway is activated (5).

Powers and co-workers (6) showed that retrograde (RTG) target genes can also be activated by exposure of cells to rapamycin, an inhibitor of the target of rapamycin (TOR) kinase signaling pathway that is involved in a broad range of cellular activities, including growth control and nutrient sensing (7). Inhibition of TOR mimics nutrient starvation, resulting in the activation of genes of both carbohydrate and nitrogen metabolism (8). Mitochondrial function and TOR signaling affect the activity of Mks1p, a negative regulator of the RTG pathway (5, 6, 9–12), by influencing its interaction with Rtg2p or with the 14-3-3 proteins Bmh1p and Bmh2p (13, 14), when bound to Bmh1p and Bmh2p, Mks1p inhibits RTG-dependent gene expression and when bound to Rtg2p, Mks1p is inactive (9).

The WD-40 protein, Lst8p, is also a negative regulator of the RTG pathway (15, 16). Analysis of different mutant alleles of LST8 that result in constitutive activation of RTG target gene expression reveals that Lst8p acts at two distinct sites, one upstream and the other downstream of Rtg2p (15, 16). The former site is believed to involve the role of Lst8p on the activity or assembly of the SPS (Ssy1p, Ptp3p, and Ssy5p) amino acid-sensing system, affecting the ability of cells to sense external glutamate (17). How Lst8p negatively regulates the RTG pathway downstream of Rtg2p is unclear. Interestingly, Lst8p is also a component of the TOR complexes (16, 18, 19). GBL, the mammalian homologue of the yeast Lst8p, has been shown to bind to the kinase domain of mammalian TOR and to be needed, together with raptor (the homologue of yeast Kog1p), for formation of the nutrient-sensitive complex that signals to the cell growth machinery (20, 21).

Inhibition of TOR results in the activation of expression of genes encoding enzymes for the synthesis of glutamate and glutamine as well as the activities of transporters responsible for the uptake of amino acids for use as a source of nitrogen. The RTG pathway has been shown to be regulated by glutamate, glutamine, and ammonia (2, 11, 22, 23). When cells are provided with a poor nitrogen source, such as proline, or when
the supply of a good nitrogen source becomes limiting, the GATA transcription factors Gln3p and Gat1p accumulate in the nucleus and activate transcription of genes of the nitrogen catabolite repression (NCR) pathway, such as GLN1, whose expression is regulated by Gln3p, and DAL5, a target of Gat1p (24–30). The same effect is elicited by treating cells with rapamycin (31–33). Glucose starvation has also been reported to cause nuclear localization of Gln3p and increased NCR-sensitive transcription, depending upon the nitrogen source provided (26, 34).

It has been suggested that TOR is a direct regulator of the RTG pathway (6, 22, 23). Hence, to better understand the molecular mechanisms by which cells sense changes in carbon and nitrogen sources as well as the role of mitochondria in the integration of these metabolic signals, we have investigated how the retrograde and TOR signaling pathways integrate in response to specific stimuli in yeast cells. Our results show that the two pathways can be separated such that RTG responses can occur independent of TOR signaling.

**MATERIALS AND METHODS**

**Strains, Media, Growth Conditions, and General Methods**—Yeast strains used are reported in **TABLE ONE** and were grown at 30 °C in YPD, YPR, YPGly (1% yeast extract, 2% bactopeptone, and 2% glucose, respectively), YNBc/D/R (0.67% yeast nitrogen base, 1% casamino acids, 2% dextrose/raffinose) or in minimal YNB/D/R medium (0.67% yeast nitrogen base, 2% dextrose/raffinose) supplemented with the necessary auxotrophic requirements with or without glutamate at the concentrations indicated under "Results" and in the figures. Rapamycin (Sigma) was dissolved in 90% ethanol/10% Tween 20 and added to yeast cell cultures grown to mid-log phase and subsequently plated on solid YPD medium. Single colonies inviable on YPGly solid medium were selected and analyzed for complete mitochondrial DNA depletion by 4'-6'-diamino-2-phenylindole staining and epifluorescence microscopy analysis. Strains carrying lst8–1, lst8–2, lst8–4, and lst8–5 mutations (15) were obtained by replacing the wild-type LST8 gene with respective lst8 mutant genes by two-step gene replacement. Deletion of GLN3 and GAT1 genes was performed by PCR amplification of the respective kanMX4 deletion cassette from the commercially available Research Genetics strain (Invitrogen). Genomic DNA was isolated from gln3Δ::kanMX4/GLN3 and gat1Δ::kanMX4/GAT1 cells and amplified using the oligonucleotide couples 5'-GTGACATG-GCAAATGCT-3' and 5'-CCAAGC-TCAAACAGCA-3', respectively. The PCR products were then used to replace the GLN3 and GAT1 wild-type genes in S288c cells, and the replacement was confirmed by Southern blotting analysis of genomic DNA digested with HpaI/Sacl and SalI/BglII, respectively. To perform gln3Δ::gat1Δ double knock-out, a gln3Δ::URA3 deletion cassette was constructed. gln3Δ::kanMX4 module was amplified from the genomic DNA isolated from the previously constructed gln3Δ::kanMX4 strain using the oligonucleotides 5'-GGATCCCTCGAGC-3' and 5'-GGATGACATG-GCAAATGCT-3'. Southern blotting analysis of genomic DNA digested with HpaI/Sacl and SalI/BglII, respectively. To perform gln3Δ::gat1Δ double knock-out, a gln3Δ::URA3 deletion cassette was constructed. gln3Δ::kanMX4 module was amplified from the genomic DNA isolated from the previously constructed gln3Δ::kanMX4 strain using the oligonucleotides 5'-GGATCCCTCGAGC-3' and 5'-GGATGACATG-GCAAATGCT-3'.

**TABLE ONE**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| S288c  | MATa ura3Δ–52 | Butow laboratory collection |
| PSY142 | MATa ura3Δ leu2Δ lys2Δ CIT2-ΔlacZ | Butow laboratory collection |
| ZLY98407 | MATa ura3Δ–52 CIT2::kanMX4::CIT2-ΔlacZ lst8–1 | This study |
| ZLY98573 | MATa ura3Δ–52 CIT2::kanMX4::CIT2-ΔlacZ lst8–2 | This study |
| ZLY98405 | MATa ura3Δ–52 CIT2::kanMX4::CIT2-ΔlacZ lst8–4 | This study |
| ZLY98577 | MATa ura3Δ–52 CIT2::kanMX4::CIT2-ΔlacZ lst8–5 | This study |
| ZLY98832 | MATa ura3Δ–52 CIT2::kanMX4::CIT2-ΔlacZ lst8–5 rtg3Δ::kanMX4 | This study |
| SGY127 | MATa ura3Δ–52 gat1Δ::kanMX4 | This study |
| SGY121 | MATa ura3Δ–52 gln3Δ::kanMX4 | This study |
| SGY164 | MATa ura3Δ–52 gat1Δ::kanMX4 gln3::URA3 | This study |
| BY741 | MATa ura3ΔΔ leu2ΔΔ his3ΔΔ met15ΔΔ | ATCC |
| JTY431 | MATa ura3ΔΔ leu2ΔΔ his3ΔΔ met15ΔΔ sit4::kanMX4 | This study |
| ZLY98711 | MATa ura3ΔΔ leu2ΔΔ his3ΔΔ met15ΔΔ pph21Δ::kanMX4 pph22Δ::kanMX4 | This study |
| ZLY98713 | MATa ura3ΔΔ leu2ΔΔ his3ΔΔ lys2ΔΔ pph21Δ::kanMX4 sit4Δ::kanMX4 | This study |
| ZLY98715 | MATa ura3ΔΔ leu2ΔΔ his3ΔΔ lys2ΔΔ met15ΔΔ pph22Δ::kanMX4 sit4Δ::kanMX4 | This study |
| TSY279 | MATa ura3ΔΔ leu2ΔΔ lys2ΔΔ RTG3-GFP | (5) |
| TSY328 | MATa ura3ΔΔ leu2ΔΔ rtg2Δ::LEU2::RTG3-GFP | (5) |
| TSY1112 | MATa ura3ΔΔ leu2ΔΔ mks1ΔΔ::LEU2::RTG3-GFP | (5) |
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(0.15 M NaCl, 0.15 M sodium citrate) and cross-linked to the membrane with a Stratalink® UV-cross-linker (Stratagene). Radiolabeled probes were added and hybridized overnight at 65 °C. [α-32P]dCTP (3000 Ci/mmol, PerkinElmer Life Sciences)-labeled DNA probes for hybridization were prepared by using the Random Primed DNA labeling kit (Roche Applied Science). Blots were exposed on Hyperfilm-MP (Amersham Biosciences). The probes used for confirming GLN3 and GAT1 deletions were the smallest Sall digestion products of the PCR-amplified products containing gln3∆:kanMX4 and gat1∆:kanMX4 modules (see above), respectively.

Total yeast RNA was isolated as previously described (37) from 50–200 ml of liquid culture (A600 = 0.7–0.8). RNA was aliquotted and stored at −80 °C. For Northern blot analysis 40 μg of RNA were separated by electrophoresis on a 1.5% agarose gel containing 6% formaldehyde for 3 h at room temperature. The fractionated RNAs were transferred to a nylon membrane and hybridized with 32P-labeled DNA probes as previously described for Southern blot analysis. Hybridized blots were scanned with a Storm 820 PhosphorImager (Amersham Biosciences) or exposed on Hyperfilm-MP (Amersham Biosciences). For quantitative Northern analysis radioactive band images were quantified against the levels of actin mRNA using ImageQuant 5.0 software (Amersham Biosciences). The DLD3, GLN1, DAL5, and ARG4 probes were amplified by PCR from selected regions of the respective genes using genomic DNA as template. The CIT2 and ACT1 probes were prepared as previously described (38).

The GFP-tagged fusions of GLN3 and GAT1 were constructed using the primer couples 5′-GGATCCACTAGTTGACGTCAACTCCATAA-3′, 5′-GGATCCGGGTTAGCAGGTAAGCCGTCAACA-3′, and 5′-GGATCCCATGAATTCGCTAGGTAAGCCGTCAACA-3′, respectively, with restriction sites used for cloning underlined. 2960- and 2200-bp fragments digested with SpeI/SalI or XbaI/SalI, and cloned into pRS416-GFP plasmid (9), yielding pRS416-GLN3-GFP and pRS416-GAT1-GFP. The GFP-tagged fusions of GLN3 and GAT1 were constructed using the primer couples 5′-GGATCCACTAGTTGACGTCAACTCCATAA-3′, 5′-GGATCCGGGTTAGCAGGTAAGCCGTCAACA-3′, and 5′-GGATCCCATGAATTCGCTAGGTAAGCCGTCAACA-3′, respectively, with restriction sites used for cloning underlined. 2960- and 2200-bp fragments digested with SpeI/SalI or XbaI/SalI, and cloned into pRS416-GFP plasmid (9), yielding pRS416-GLN3-GFP and pRS416-GAT1-GFP.

Fluorescence Microscopy Analysis—Cells expressing Gln3p-GFP or Gat1p-GFP proteins as indicated in Fig. 5 were grown in YNBD medium with 0.2% glutamate to A600 = 0.6–0.8 and incubated with 300 ng/ml rapamycin or 90% ethanol/10% Tween 20 as a control for 20 min at 30 °C as described above. Cells expressing Rtg1p-GFP fusion protein were grown in YNBD medium with 0.2% glutamate to A600 = 0.6–0.8 and treated with or without 50 mM 3-AT for 30 min at 30 °C. Cells were concentrated by pelleting, and live cells were observed by fluorescence microscopy on a Leica microscope (model DMRXE) equipped with an HBO 100 W/2 mercury arc lamp, >100 Plan-Apochromat objective. Epifluorescence was detected with a green fluorescence filter set (Leica N2.1–513812). Digital images were acquired with a Hamamatsu C5810 cooled CCD camera.

RESULTS

Separation of the Retrograde and TOR Pathways—The retrograde pathway was originally defined in comparisons of gene expression between respiratory-competent ρ+ cells and respiratory-incompetent petite cells grown in rich medium (39). To gain insight into how the retrograde and TOR signaling pathways are integrated under these growth conditions, we examined the expression of two RTG target genes, CIT2 and DLD3, and two NCR-responsive genes, GLN1 and DAL5, in ρ+ and ρ− cells before and after the addition of rapamycin to the medium. Characteristic of the retrograde response to mitochondrial dysfunction, CIT2 and DLD3 expression is higher in ρ− than in ρ+ cells (Fig. 1, lanes 1 and 3). In contrast, GLN1 expression was only slightly increased in ρ− cells, and no DAL5 expression was detected in either ρ+ or ρ− cells. Rapamycin treatment of ρ− cells resulted in large increases in the expression of all four genes (lanes 1 and 2), in agreement with the findings of Komel et al. (6). However, there was no substantial increase in expression of these genes in rapamycin-treated ρ+ cells (lane 4), and DAL5 expression decreased somewhat under these conditions.

Glutamate is a repressor of RTG-dependent gene expression (2). Thus, CIT2 expression was analyzed in cells grown in minimal medium containing 0.5% ammonium sulfate with or without 0.1% glutamate. A high level of CIT2 expression was observed in ρ− cells due to glutamate starvation (Fig. 2, lane 1), with no increased expression detected in ρ+ cells (lane 2). The addition of 0.1% glutamate to the culture medium abolished CIT2 expression in both ρ+ and ρ− cells (lanes 3 and 4). Rapamycin treatment resulted in a 8-fold increase in CIT2 expression in both ρ+ and ρ− cells grown in the absence of glutamate (lanes 1, 2, 5, and 6), whereas in the presence of glutamate in the medium, CIT2 expression increased 30- to 40-fold following TOR inhibition by rapamycin (lanes 3, 4, 7, and 8).

Lst8 Mutations Increase the Expression of CIT2 and GLN1, but Not DAL5—A common feature of the RTG and TOR pathways is Lst8p. Biochemical studies have shown that Lst8p is an integral component of the TOR complexes, and it has been proposed to act as a positive regulator of TOR kinase activity (16, 18, 19, 40). Genetic studies indicate that Lst8p is a negative regulator of the RTG pathway functioning at two sites, one upstream and the other downstream of Rtg2p (15). These regulatory sites have been revealed through the identification and analysis of collections of lst8 mutants. The lst8–1 mutant allele, for example, shows an RTG2-dependent activation of CIT2 expression, which is also observed in mutants defective in the SPS (Ssy1p, Prr3p, and Ssy5p) amino acid-sensing system (15). It has been proposed that lst8–1 mutant cells are defective in targeting Ssy1p to the plasma membrane. As a consequence, lst8–1 mutant cells would behave as though they were starved for glutamate and thus up-regulate the RTG pathway because of an inactive SPS (Ssy1p, Prr3p, and Ssy5p) sensor system.
To investigate how Lst8p affects the link between RTG and TOR signaling, we compared the expression of CIT2, GLN1, and DAL5 in ρ+ wild-type and four lst8 mutants strains harboring the lst8–1 and lst8–2, lst8–4, and lst8–5 mutant alleles; the latter three mutants show RTG2-independent activation of CIT2 expression (15). These cells were grown in rich raffinose medium with or without the addition of rapamycin (Fig. 3). As expected, CIT2 expression was increased in all the lst8 mutant strains, although to somewhat different extents depending on the lst8 allele. The addition of rapamycin had little effect on the level of CIT2 expression in lst8–2, lst8–4, and lst8–5 cells but resulted in a significant increase in CIT2 expression in lst8–1 cells.

Among the lst8 mutants, GLN1 was most dramatically activated in the lst8–5 mutant, and the differential levels of GLN1 expression observed among all of the lst8 mutants tested was not substantially affected by the addition of rapamycin. These data, in agreement with the findings of Chen and Kaiser (16), further suggest a complex interaction of Lst8p with TOR in that the effect of rapamycin is not a simple consequence of abrogation of a positive regulatory effect of Lst8p on TOR activity. An unexpected finding was that none of the lst8 mutants caused a detectable increase in the expression of DAL5, although both DAL5 and GLN3 are induced by rapamycin treatment. Finally, inactivation of RTG3 completely abolished the expression of CIT2 but had no effect on GLN1 and DAL5 expression in lst8–5 mutant cells in the absence or presence of rapamycin (Fig. 3, lanes 6 and 12), suggesting that failure to activate DAL5 expression in lst8 mutants is not due to activation of the RTG pathway. Together, these data indicate that lst8 mutants differentiate among targets regulated by TOR.

Both Gat1p and Gln3p Are Essential for Rapamycin-induced Expression of DAL5—NCR-sensitive gene expression in yeast has been shown to be mediated mainly by two GATA family transcriptional activators, Gln3p and Gat1p (24, 25). With the aim of elucidating the molecular correlates of the mechanism of GLN1 and DAL5 expression, S288c yeast cells harboring single or double deletions of GLN3 and GAT1 were grown in YPR medium and analyzed for rapamycin-induced expression of CIT2, GLN1, and DAL5 (Fig. 4). Rapamycin-triggered increase in CIT2 expression was virtually unaffected by gln3Δ or gat1Δ single and double deletion mutations (lanes 1–8), because its transcription depends exclusively on Rtg1/3p. In the experimental conditions used, deletion of GLN3 and/or GAT1 did not greatly affect GLN1 up-regulation by rapamycin (lanes 1–8), suggesting that regulation of GLN1 expression is complex. Three separate systems have been identified that control the transcription of GLN1 (41): the URE2/GLN3 system, GCN4, and the system that responds to purine starvation. The regulation elicited by these systems has different strengths as judged by in vitro reporter gene analysis of the GLN1 promoter region (41) and is supported by a mathematical model developed to describe the expression of the genes involved in nitrogen metabolism (42). Moreover, the presence of raffinose in the culture medium used in our experiments has been shown to trigger nuclear localization of Gln3p (26, 34). Thus, GATA-dependent regulation of GLN1 expression appears to be dispensable under the experimental conditions analyzed. By contrast, increased DAL5 expression upon TOR inhibition by rapamycin (Fig. 4, lanes 1 and 5) is completely dependent on both Gln3p and Gat1p (lanes 2–4, 6–8). Our current results and previous findings by Chen and Kaiser (16) clearly showed that lst8 mutations up-regulate the expression of the NCR target genes GAP1 and GLN1, whose expression has been shown to be dependent on Gln3p. The strict dependence of DAL5 expression on both Gln3p and Gat1p raises the possibility that lack of effects of lst8 mutations on DAL5 expression is due to failure of activation of Gat1p in the lst8 mutants.

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FIGURE 2. Glutamate represses CIT2 expression triggered by mitochondrial dysfunction, but not that elicited by TOR inhibition by rapamycin. RNA was extracted from ρ− and ρ+ S288c yeast strains grown in YNBd with or without 0.1% glutamate to mid-log phase and treated with rapamycin as described in the legend to Fig. 1. CIT2 mRNA levels were analyzed by Northern blotting.

FIGURE 3. Effect of lst8 mutations and TOR inhibition on RTG- and GATA factor-dependent transcription activation. CIT2, GLN1, and DAL5 expression was analyzed by Northern blotting of RNA isolated from yeast cells harboring lst8–(1–5) mutant alleles (see “Results”) or lst8–5rtg3ΔΔ and their wild-type isogenic strain grown in YPR medium before and after rapamycin administration as described in the legend to Fig. 1.

FIGURE 4. Effect of GLN3 and/or GAT1 deletion on TOR-regulated RTG- and GATA factor-dependent gene expression. Cultures of wild-type or mutant S288c were grown to mid-log phase in YPR medium with or without the addition of rapamycin as indicated and as described in the legend to Fig. 1. RNA was isolated and the levels of CIT2, GLN1, and DAL5 transcripts measured by Northern blotting.

lst8p Negatively Regulates Nuclear Localization of Gln3p but Not of Gat1p—To investigate further the differential effects of the lst8 mutants on GLN1 and DAL5 expression, we analyzed the intracellular localization of Gln3p and Gat1p in S288c wild-type and isogenic lst8–5 mutant cells in the presence or absence of rapamycin using C-terminal GFP-tagged derivatives of Gln3p and Gat1p (Fig. 5). In wild-type cells, Gln3p-GFP was mainly, but not exclusively, localized to the cytoplasm and the distribution of fluorescence was not uniform. This “clumpy” fluorescence distribution is reminiscent of the non-uniform distribution of Gln3p in the cytoplasm of glutamine-grown cells, which has been argued to derive from its localization to components of the NCR pathway (26). After rapamycin treatment Gln3p-GFP localized to the
nucleus in the majority of \textit{lst8–5} and wild-type cells. Interestingly, in the absence of rapamycin, a uniform distribution of Gat1p-GFP-derived fluorescence in the cytoplasm was observed both in \textit{lst8–5} and wild-type cells, showing exclusion of Gat1p from the nucleus. Rapamycin treatment of both wild-type and \textit{lst8–5} mutant cells caused nuclear localization of Gat1p-GFP. These data are in agreement with the observed level of GLN1 and DAL5 expression in cells harboring the \textit{lst8–5} mutant allele (Fig. 3) and give clear evidence that Lst8p is a negative regulator of Gln3p, as well as of Rtg1p, but not of Gat1p. Only TOR inactivation by rapamycin triggers Gat1p translocation to the nucleus, resulting in Gln3p/Gat1p-dependent DAL5 expression, apparently through a branch of TOR signaling that does not involve Lst8p.

The role of the TOR proteins in yeast has been shown to be mediated, at least in part, by two functionally redundant catalytic subunits of type 2A and type 2A-like phosphatases, encoded by \textit{pph21} and \textit{pph22}, and by a 2A-like phosphatase encoded by \textit{SIT4} (31, 43–45). We compared RTG-dependent and NCR-sensitive gene expression in \textit{sit4Δ, sit4Δ, pph21Δ, sit4Δ, pph22Δ, and pph21Δ, pph22Δ} yeast cells grown in YPD medium before and after TOR inhibition by rapamycin (Fig. 6). A \textit{sit4Δ} mutation alone partially inhibited rapamycin-induced \textit{CIT2} expression, and combination with \textit{pph21Δ} or \textit{pph22Δ} does not further inhibit \textit{CIT2} expression (Fig. 6). However, \textit{pph21Δ, pph22Δ} double mutations inhibited rapamycin-induced \textit{CIT2} expression to the same extent as the \textit{sit4Δ} single mutation. Deletion of \textit{SIT4} alone, or together with \textit{PPH21} or \textit{PPH22}, caused a decrease in DAL5 induction by rapamycin but had little effect on GLN1 expression. However, deletion of both \textit{PPH21} or \textit{PPH22} caused a significant reduction of \textit{GLN1} induction and complete abolishment of DAL5 induction triggered by TOR inactivation. These data confirm that Lst8p is positively acting in the TOR pathway as suggested by Chen and Kaiser (16) and show additionally that Lst8p and TOR may act independently, at least in the signaling branch, which regulates nutrient-sensing gene expression with that of TOR-regulated transcription mediated by type 2A and type 2A-like phosphatases.

\textit{Histidine Starvation Activates the RTG, but Not the NCR Pathway—}Recently, it has been suggested that intracellular glutamate, but not glutamine, inhibits the retrograde pathway under the control of the TOR kinases (22). Those results were based on observations that treatment of cells with MSX, an inhibitor of glutamine synthetase (46), results in glutamine starvation and concomitant activation of \textit{CIT2} expression in cells grown in medium containing glutamate, as well as on large-scale genetic analyses converging on glutamine as a key molecule that may act upstream of TOR (22, 47). To determine whether activation of \textit{CIT2} expression is specific to glutamine starvation, we examined \textit{CIT2} expression in cells starved for other amino acids, for example, histidine. Histidine starvation in yeast can be easily achieved by treating cells with 3-AT, a competitive inhibitor of His3p (48, 49). Fig. 7A shows that treatment of cells with 50 mM 3-AT for 30 min completely abolishes glutamate repression of \textit{CIT2} expression. Consistent with previously published results (22), 2 mM MSX treatment for 30 min also results in high \textit{CIT2} expression in cells grown in the presence of glutamate. By contrast, 3-AT treatment has no effect on \textit{GLN1} expression although glutamine starvation due to MSX treatment results in high \textit{GLN1} expression. Expression of \textit{ARG4}, whose expression is under the control of amino acid starvation pathway, is induced by both MSX and 3-AT treatments. Addition of histidine to the growth medium can efficiently block 3-AT-induced \textit{CIT2} and \textit{ARG4} expression (data not shown), indicating that the 3-AT effect observed in Fig. 7A is largely due to histidine starvation. The differential effects of 3-AT on expression of \textit{CIT2}, a target of the RTG pathway, and of \textit{GLN1}, a target of the NCR pathway, suggest that regulation of these two pathways by TOR kinases is complex.

To determine whether 3-AT activation of \textit{CIT2} expression is due to Rtg1/3p activation, we examined the intracellular localization of a functional Rtg3p-GFP fusion. As reported previously, Rtg3p-GFP was localized in the cytoplasm in glutamate-grown cells and an \textit{mks1Δ} mutation resulted in its constitutive nuclear localization and concomitant activation of the RTG pathway (Fig. 7B and Ref. 5). 3-AT treatment for 30 min resulted in strong Rtg3p-GFP nuclear localization similar to that in which the RTG pathway is constitutively activated in \textit{mks1Δ} mutant cells. The 3-AT-induced nuclear localization of Rtg3p-GFP is dependent on Rtg2p, indicating that histidine starvation activates the RTG pathway upstream of Rtg2p. Histidine starvation is known to activate the general amino acid control pathway mediated by the Gcn2p kinase and the Gcn4p transcription factor (50). To determine whether histidine starvation activates Rtg1/3p through the general amino acid control pathway, we examined the effects of a dominant Gcn4 mutant, \textit{GCN4F} (51) and two constitutively activated \textit{GCN2} mutants, \textit{GCN2F}–S16 (E532K, E1522K) and \textit{GCN2F}–S13 (M719V, E1537G) (52) on the expression of a \textit{CIT2-lacZ} reporter gene. These mutants have been
shown to constitutively activate the general amino acid control pathway. Fig. 7C shows that none of these mutants activates CIT2-lacZ expression in cells grown in minimal medium supplemented with 0.2% glutamate. Thus, histidine starvation activates the RTG pathway independent of the general amino acid control pathway.

**DISCUSSION**

In the current work we have explored the link between TOR-mediated control of Rtg1/3p in response to nutrient starvation and the retrograde response to mitochondrial dysfunction. To these ends, we investigated the effects of TOR inhibition by rapamycin and different lst8 mutants on the expression of RTG target and NCR-sensitive genes in respiratory-competent and respiratory-deficient yeast cells. Several lines of evidence support the conclusion that the retrograde response to mitochondrial dysfunction is separable from TOR control of expression of NCR-responsive genes, as summarized diagrammatically in Fig. 8. First, the level of induction of the expression of CIT2, DL3D3, GLN1, and, partly, DAL5 as a result of inhibiting TOR with rapamycin is independent of the mitochondrial respiratory state, whereas RTG-dependent gene expression is specifically higher in rH9267 than in rH11001 cells (Fig. 1). Moreover, of relevance is the observation that glutamate represses CIT2 expression in response to mitochondrial dysfunction but not that induced by rapamycin treatment (Fig. 2). Transcription factors regulating the two sets of genes analyzed do not functionally overlap, as shown in this (Figs. 3, 4) and other studies (6, 23). Thus, it is likely that intracellular metabolite levels control RTG gene expression through either retrograde or TOR-dependent pathways.

Second, our results show that both glutamine and histidine starvation after MSX and 3-AT treatment, respectively, increase CIT2-lacZ expression. Glutamine starvation activates Gln3p and Rtg1/3p, but not Gat1p, whereas histidine starvation only activates Rtg1/3p. Integration of Retrograde and TOR Signaling
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(22) 3-AT treatment activates expression of CIT2, an RTG target gene, but not GLN1, a Gin3p target gene. This raises the question whether glutamine is the metabolite signaling to the TOR pathway as recently proposed by Hall and co-workers (22). 3-AT elicits histidine starvation, but not

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GLN3, but not GAT1, and this inhibition is downstream of SIT4 (54). Interestingly, activation of GLN3 in npr1 mutants requires the ubiquitination machinery when cells are grown on a poor nitrogen source such as proline but not when TOR is inhibited by rapamycin treatment (54).

Thus, transcription factors regulated by TOR can also be differentially regulated by multiple factors through parallel pathways, such as the RTG pathway in the case of Rtg1/3p, and the ubiquitin-dependent signaling pathway in the case of Gin3p (54). Together with previous evidence showing that Lst8p negatively regulates RTG gene function at two sites, one upstream and one downstream of Rtg2p (15), these data strongly suggest that Lst8p may have a role in connecting the RTG and TOR pathways.

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