Rtg1p and Rtg3p are two basic helix–loop–helix, retrograde transcription factors in the budding yeast *Saccharomyces cerevisiae*. Both factors heterodimerize to activate the transcription of nuclear genes in response to mitochondrial dysfunction and glutamate auxotrophy, but are not well characterized in other yeasts. Here, we demonstrate that the Rtg1p/Rtg3p-mediated retrograde signaling pathway is absent in the methylotrophic yeast *Pichia pastoris*. We observed that *P. pastoris* Rtg1p (PpRtg1p) heterodimerizes with *S. cerevisiae* Rtg3p and functions as a nuclear, retrograde transcription factor in *S. cerevisiae*, but not in *P. pastoris*. We noted that *P. pastoris* Rtg3p lacks a functional leucine zipper and interacts with neither *S. cerevisiae* Rtg1p (ScRtg1p) nor PpRtg1p. In the absence of an interaction with Rtg3p, PpRtg1p has apparently acquired a novel function as a cytosolic regulator of multiple *P. pastoris* metabolic pathways, including biosynthesis of glutamate dehydrogenase 2 and phosphoenolpyruvate carboxykinase required for the utilization of glutamate as the sole carbon source. PpRtg1p also had an essential role in methanol metabolism and regulated alcohol oxidase synthesis and was required for the metabolism of ethanol, acetate, and oleic acid, but not of glucose and glycerol. Although PpRtg1p could functionally complement ScRtg1p, ScRtg1p could not complement PpRtg1p, indicating that ScRtg1p is not a functional PpRtg1p homolog. Thus, PpRtg1p functions as a nuclear, retrograde transcription factor in *S. cerevisiae* and as a cytosolic, post-transcriptional regulator in *P. pastoris*. We conclude that PpRtg1p is a key component of a signaling pathway that regulates multiple metabolic processes in *P. pastoris*.

Retrograde (RTG)\(^3\) response is an interorganellar signaling pathway that maintains homeostasis under normal growth conditions and facilitates adaptation of eukaryotic cells to various stresses such as mitochondrial dysfunction and nitrogen starvation among others (1, 2). RTG response, discovered in *Saccharomyces cerevisiae*, has been studied in a wide range of organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse, and humans (3). In *S. cerevisiae*, genes of the citric acid cycle are repressed during glucose metabolism, and therefore *CIT1*, *ACO1*, and *IDH1* encoding the first three enzymes of the citric acid cycle (mitochondrial citrate synthase, aconitate, and isocitrate dehydrogenase, respectively) and *CIT2* encoding peroxisomal citrate synthase are up-regulated by activators of the RTG pathway, resulting in the synthesis of α-ketoglutarate, the precursor for glutamate biosynthesis (2). RTG response is also triggered by mitochondrial dysfunction and is well studied in the petite (Rho\(^-\)) mutants of *S. cerevisiae*. The key event in *S. cerevisiae* RTG signaling is the translocation of Rtg1p–Rtg3p, a heterodimeric, basic helix–loop–helix/leucine zipper transcription factor, from the cytoplasm to the nucleus. Inside the nucleus, it binds to the R box (5’-GTAC-3’) of promoters of RTG response genes such as *CIT2*, *CIT1*, *ACO1*, and *IDH1* and activates their transcription (4). Although only *S. cerevisiae* Rtg3p (ScRtg3p) possesses a transcriptional activation domain, heterodimerization with ScRtg1p is essential for DNA binding and transactivation. Nucleocytoplasmic trafficking of ScRtg1p/ScRtg3p is regulated by complex interactions involving several proteins, including Rtg2p, Mks1p, and Bmh1p/2p (5). When phosphorylated, Mks1p complexes with Bmh1p/2p, resulting in the sequestration of Rtg1p–Rtg3p heterodimer in the cytoplasm. Rtg2p, a cytoplasmic protein with an N-terminal ATP-binding domain competes for Bmh1p/2p binding to Mks1p, thereby relieving cytoplasmic sequestration and facilitating nuclear translocation of Rtg1p/3p. Release of Mks1p from Bmh1p/2p is associated with reduced phosphorylation of Mks1p. Mks1p–Rtg2p interaction is inhibited by ATP at a concentration of 3–4.5 mM, suggesting that the RTG pathway may also be involved in ATP homeostasis (6). Free Mks1p is degraded by Grr1p, a component of ubiquitin protein ligase. Rtg1p–Rtg3p function is also modulated by the target of the rapamycin (TOR) and the Hog1p-mediated osmoregulatory signaling pathways (7–9).
Post-transcriptional regulation by Rtg1p

Table 1
S. cerevisiae proteins involved in retrograde response and their putative P. pastoris homologues

| Proteins involved in retrograde response in S. cerevisiae | Putative P. pastoris homologues | Amino acid identity | P. pastoris gene ID |
|----------------------------------------------------------|---------------------------------|---------------------|---------------------|
| ScRtg1p (CAA90077.1)                                     | PpRtg1p (XP_002490029)         | %                   | PAS_chr1-1_0371     |
| ScRtg2p (CA96972.1)                                      | PpRtg2p (XP_002492678)         | 48                  | PAS_chr3-0452       |
| ScRtg3p (CA984929.1)                                     | PpRtg3p/PpRtgXp (AOA70166)*    | 44                  | PP7435_chr4-0030    |
| ScMks1p (Q35770.1)                                      | PpMks1p (XP_002493426)         | 46                  | PAS_chr4-0019       |
| ScVes1p (P47002.2)                                      | PpVes1p (XP_002491635)         | 34                  | PAS_chr2-1_0707     |
| ScPrt3p (P43606.1)                                      | PpPrt3p (XP_00249210)          | 47                  | PAS_chr1-0099       |
| ScTor1p (CA52849.4)                                     | PpTor1/2p (XP_002491471)       | 55                  | PAS_chr2-1_0557     |
| ScTor2p (CA50548.1)                                     | PpTor1/2p (XP_002491471)       | 57                  | PAS_chr2-1_0557     |
| ScLrt8p (NP_014392.3)                                   | PpLrt8p (XP_002492056)         | 78                  | PAS_chr2-2_0150     |
| ScBmh1p (CAA6959.1)                                     | PpBmh1/2p (XP_002490987)       | 83                  | PAS_chr2-1_0809     |
| ScBmh2p (CA59275.1)                                     | PpBmh1/2p (XP_002490987)       | 82                  | PAS_chr2-1_0809     |
| ScMks1p (CA882527.1)                                    | PpMks1p (XP_002493871)         | 29                  | PAS_chr4-0962       |

* This protein, annotated as PpRtg3p (http://www.uniprot.org/uniprot/FQ2XT2), does not heterodimerize with Rtg1p and hence is designated as PpRtgXp in this study.

RTG signaling has not been well characterized in yeasts other than S. cerevisiae. In Candida albicans, Rtg3p was shown to be involved in tolerance to cations and antifungal drugs as well as serum-induced filamentation (10). However, its interaction with Rtg1p and its role in glutamate homeostasis have not been investigated. In other yeasts, most studies are focused on the ability of putative RTG homologues to complement corresponding mutations in S. cerevisiae. For example, Rtg2p and Mks1p homologues from Kluyveromyces lactis and Klyveromyces waltii can complement ∆rtg2 and ∆mks1 mutations in S. cerevisiae, and ATP-mediated dissociation of Mks1p from Rtg2p was shown to be conserved among these yeasts as well (6). Putative RTG2 genes from Candida glabrata, Ashbya gossypii, K. lactis, and Vanderwaltozyma polyspora functionally complement S. cerevisiae ∆rtg2 mutant (11). Pichia pastoris (Komagataella phaffii), a methylophytic yeast, possesses a potent, methanol-inducible promoter encoding alcohol oxidase I (AOXI) and grows to very high cell densities by virtue of its respiratory metabolism (12, 13). It is widely used for the production of several recombinant proteins (14, 15). In this study, we demonstrated that Rtg3p is absent in P. pastoris and that Rtg1p functions as a cytosolic regulator rather than a nuclear, retrograde transcription factor. We conclude that the classical mitochondrial retrograde response involving Rtg1p–Rtg3p heterodimer is not functional in respiratory yeasts such as P. pastoris and that Rtg1p has evolved as an Rtg3p-independent regulator of multiple metabolic pathways.

Results
Rtg3p is absent in P. pastoris, and PpRtg1p is a functional homologue of ScRtg1p

The lack of information on mitochondrial retrograde signaling in yeasts other than S. cerevisiae prompted us to investigate this pathway in P. pastoris, a respiratory and methylotrophic yeast of biotechnological importance. BLAST analysis of the P. pastoris genome database using amino acid sequences of ScRtg proteins as the query indicated the existence of putative P. pastoris Rtg (PpRtg) homologues (Table 1). Because RTG signaling culminates in the activation of nuclear genes by the heterodimeric transcription factor Rtg1p–Rtg3p, we focused our attention on these two proteins in this study. The basic helix–loop–helix domains of P. pastoris proteins annotated as PpRtg1p and PpRtg3p share 48 and 44% amino acid identity with those of ScRtg1p and ScRtg3p, respectively (Fig. 1A and Table 1). PpRtg3p is designated as PpRtgXp in this study (see Table 1) because the leucine zipper essential for heterodimerization with Rtg1p is not conserved in this protein (Fig. 1B) (16, 17). To examine the ability of PpRtg1p and PpRtgXp to heterodimerize with each other as well as with ScRtg homologues, these proteins were expressed as maltose-binding protein (MBP) or GSH transferase (GST) fusion proteins in Escherichia coli (Fig. 1C), and protein–protein interactions were studied. GST-Rtg1p bound to GSH beads was incubated with E. coli cell lysates containing MBP-Rtg3p or MBP-RtgXp. After washing, proteins retained on the beads were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue R staining. Although PpRtg1p and ScRtg1p interacted with ScRtg3p (Fig. 1D, lanes 4 and 8), PpRtgXp failed to interact with either PpRtg1p or ScRtg1p (Fig. 1D, lanes 3 and 7). Thus, PpRtgXp is unlikely to be a functional homologue of ScRtg3p.

The ability of Rtg1p–Rtg3p heterodimers to bind to radiolabeled S. cerevisiae CIT2 upstream activation sequence containing R box (4) was examined in an electrophoretic mobility shift assay (EMSA). PpRtg1p–ScRtg3p (Fig. 1E, lane 5) but not PpRtgXp–ScRtg1p (Fig. 1E, lane 7) or PpRtgXp–PpRtg1p (Fig. 1E, lane 9) interacted with CIT2 R box. A point mutation within the R box abolishes the formation of ScRtg1p–ScRtg3p (Fig. 1E, lane 4) and PpRtg1p–ScRtg3p complexes (Fig. 1E, lane 6). We generated PpRtgXp* by fusing the region containing amino acids 345–486 of ScRtg3p to the C-terminal region of PpRtgXp (Fig. 1F). When expressed as an MBP fusion protein, PpRtgXp* interacted with ScRtg1p as well as PpRtg1p (Fig. 1G, lanes 2 and 5), suggesting that the absence of a dimerization domain may be responsible for the inability of PpRtgXp to dimerize with Rtg1p. PpRtg1p and ScRtg1p were expressed as GFP fusion proteins in S. cerevisiae Δrtg1 (∆Scrtg1), and expression of PpRtg1GFP and ScRtg1GFP was confirmed by Western blotting using anti-GFP antibodies (Fig. 2A). CIT2 mRNA levels were up-regulated in ∆Scrtg1 expressing PpRtg1GFP as well as ScRtg1GFP, as evident from quantitative real-time PCR (qPCR) of RNA isolated from Scrtg1 (∆Scrtg1), and expression of Scrtg1GFP was confirmed by Western blotting using anti-GFP antibodies (Fig. 2A). CIT2 mRNA levels were up-regulated in ∆Scrtg1 expressing PpRtg1GFP as well as ScRtg1GFP, as evident from quantitative real-time PCR (qPCR) of RNA isolated from Scrtg1 (∆Scrtg1), and expression of Scrtg1GFP was confirmed by Western blotting using anti-GFP antibodies (Fig. 2A). CIT2 mRNA levels were up-regulated in ∆Scrtg1 expressing PpRtg1GFP as well as ScRtg1GFP, as evident from quantitative real-time PCR (qPCR) of RNA isolated from Scrtg1 (∆Scrtg1), and expression of Scrtg1GFP was confirmed by Western blotting using anti-GFP antibodies (Fig. 2A).
Subcellular localization studies using anti-GFP antibodies indicated that PpRtg1pGFP and ScRtg1pGFP localized to the cytosol of \( \text{Scrtg1}\) cultured in YNBD Glu medium but translocated to the nucleus when cultured under glutamate-deficient conditions (Fig. 2F). These results indicate that PpRtg1p is a functional homologue of ScRtg1p.

**Regulation of glutamate utilization pathway of \( \text{P. pastoris} \) by PpRtg1p**

Thus far, Rtg3p-independent function has not been reported for Rtg1p. The absence of Rtg3p in \( \text{P. pastoris} \) led us to investigate \( \text{PpRtg1p} \) function in detail. A \( \text{Pprtg1} \) strain was generated, and absence of \( \text{PpRTG1} \) mRNA was confirmed by reverse transcription–polymerase chain reaction (RT-PCR).
**Post-transcriptional regulation by Rtg1p**

**A**

\[ \Delta Sc-PPRTG^\text{GFP} \]
\[ \Delta Sc-SCRTG^\text{GFP} \]
\[ ScRTG1 \]
\[ PrRTG1 \]
\[ GFP \]
\[ (54) \]

**B**

\[ \Delta Scrtg1 \]
\[ \Delta Sc-PPRTG^\text{GFP} \]
\[ \Delta Sc-SCRTG^\text{GFP} \]
\[ ScRTG1 \]
\[ PrRTG1 \]
\[ GFP \]
\[ (46) \]

**C**

Relative Ct/2

mRNA levels Curves

[Glutamate +](H9004)

Coomassie Brilliant Blue R staining. At least four major proteins, designated as A, B, C, and D, were either undetectable or expressed at reduced levels in \( \Delta prrtg1 \) compared with \( GSI15 \) (Table 2). Among these \( PpRtg1p \)-regulated enzymes, \( PEPCK \) and \( GDH2 \) are essential for growth of cells in YNB Glu + medium (Fig. 2E). We conclude that ScRtg1p is not a functional homologue of PpRtg1p.

**F**

[DAPI +](H9004)

[ScRTg1 +](H9004)

[Merge](H9004)

[DAPI +](H9004)

[PrRTg1 +](H9004)

[Merge](H9004)

transcription-polymerase chain reaction (RT-PCR) (Fig. 3A). Unlike \( \Delta Scrtg1 \) (Fig. 1, D and E), \( \Delta PpRtg1p \) did not exhibit glutamate auxotrophy because its growth was similar to that of \( GSI15 \) when cultured in YNBD Glu + medium (Fig. 3B). PpRtg1p and ScRtg1p were expressed as GFP- and FLAG-tagged proteins in \( \Delta prrtg1 \), and expression was confirmed by Western blotting using anti-GFP and anti-FLAG antibodies, respectively (Fig. 3, C and D). Both proteins localized to the cytosol of cells cultured in YNBD Glu − medium (Fig. 3E). Thus, nuclear localization of Rtg1p is observed only in \( S. cerevisiae \) but not \( P. pastoris \) cultured in YNBD Glu − medium (compare Figs. 2F and 3E).

**Figure 2.** PpRtg1p is a functional homologue of ScRtg1p. A, schematic diagrams of constructs designed to express ScRtg1p and PrRtg1p in \( \Delta Scrtg1 \)as GFP fusion proteins from ScRTG1 promoter. Molecular masses (kDa) of proteins are shown in parentheses. B, analysis of expression of PrRtg1p by Western blotting using anti-GFP antibodies.  

lysates were prepared from \( S. cerevisiae \) cells cultured in YNBD Glu + medium. PGK was used as loading control. Numbers indicate molecular mass (kDa) of proteins. C, analysis of CIT2 mRNA levels by qPCR in different \( S. cerevisiae \) strains as indicated. Error bars represent S.D. (n = 2). D, analysis of the ability of ScRtg1p and PrRtg1p to reverse glutamate auxotrophy of \( \Delta Scrtg1 \) by spot assay. E, quantitation of growth of \( S. cerevisiae \) strains in YNBD Glu − and YNBD Glu + media. The data are the average of two independent experiments. F, subcellular localization of ScRTg1p and PrRTg1p in cells cultured in the presence or absence of glutamate. Mouse anti-GFP antibodies and Alexa Fluor 555–conjugated donkey anti-mouse antibodies were used. DAPI was used to stain the nucleus.

**Figure 3.** Expression of ScRtg1p and PpRtg1p in \( S. cerevisiae \). A, strain designations and analysis of expression of ScRtg1p and PpRtg1p in \( \Delta Scrtg1 \)as GFP fusion proteins from ScRTG1 promoter. Molecular masses (kDa) of proteins are shown in parentheses. B, analysis of expression of PrRtg1p by Western blotting using anti-GFP antibodies. Lysates were prepared from \( S. cerevisiae \) cells cultured in YNBD Glu + medium. PGK was used as loading control. Numbers indicate molecular mass (kDa) of proteins. C, analysis of CIT2 mRNA levels by qPCR in different \( S. cerevisiae \) strains as indicated. Error bars represent S.D. (n = 2). D, analysis of the ability of ScRtg1p and PrRtg1p to reverse glutamate auxotrophy of \( \Delta Scrtg1 \) by spot assay. E, quantitation of growth of \( S. cerevisiae \) strains in YNBD Glu − and YNBD Glu + media. The data are the average of two independent experiments. F, subcellular localization of ScRTg1p and PrRTg1p in cells cultured in the presence or absence of glutamate. Mouse anti-GFP antibodies and Alexa Fluor 555–conjugated donkey anti-mouse antibodies were used. DAPI was used to stain the nucleus.

**Table 2**

| ScRtg1p and PpRtg1p to reverse glutamate auxotrophy of Flour 555– conjugated donkey anti-mouse antibodies were used. DAPI was assay. E strains as indicated. (kDa) of proteins are shown in /H9004 diagrams of constructs designed to express ScRtg1p and PpRtg1p in J. Biol. Chem. 16650 /H9004/Lysates were prepared from /H9004 GS115– when cultured in YNBD Glu−/H9004 medium. The data are the average of two independent experiments. /H9004 numbers indicate molecular mass (kDa) of proteins, respectively (Fig. 3, C and D). Both proteins localized to the cytosol of cells cultured in YNBD Glu−/H9004 medium (Fig. 3E). Thus, nuclear localization of Rtg1p is observed only in S. cerevisiae but not P. pastoris cultured in YNBD Glu−/H9004 medium (compare Figs. 2F and 3E).

P. pastoris can utilize glutamate, aspartate, or proline as the sole source of carbon and nitrogen (18) (Table 2), and growth of \( \Delta prrtg1 \) was severely compromised when cultured in yeast nitrogen base (YNB) Glu−/H9004 medium containing glutamate as the sole source of carbon (Fig. 4A). When cultured in YNB*D Glu+ medium containing glutamate rather than ammonium sulfate as the sole source of nitrogen, \( \Delta prrtg1 \) grew normally (Fig. 4A), indicating that PpRtg1p is essential for the utilization of glutamate as the sole source of carbon but not nitrogen. Because PpRtg1p is a functional homologue of ScRtg1p (Fig. 2, D and E), we examined whether ScRtg1p is a functional homologue of PpRtg1p. Only PpRtg1p GFP but not ScRtg1p FLAG facilitated the growth of \( \Delta prrtg1 \) in YNB Glu + medium (Fig. 4B). To rule out interference from epitope tags, \( \Delta prrtg1 \) strains expressing untagged PpRtg1p and ScRtg1p were constructed (Fig. 4C). Expression of mRNA encoding untagged PpRtg1p and ScRtg1p was confirmed by semiquantitative RT-PCR (Fig. 4D). As observed with epitope-tagged proteins, untagged PpRtg1p but not ScRtg1p facilitated the growth of \( \Delta prrtg1 \) in YNB Glu + medium (Fig. 4E). We conclude that ScRtg1p is not a functional homologue of PpRtg1p.

To understand the mechanism of action of PpRtg1p, GS115 and \( \Delta prrtg1 \) were cultured in YNB Glu + medium, and cell lysates were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue R staining. At least four major proteins, designated as A, B, C, and D, were either undetectable or expressed at reduced levels in \( \Delta prrtg1 \) compared with GS115 (Fig. 4A). To confirm their differential expression, these proteins were expressed as Myc- or His-tagged proteins in GS115 and \( \Delta prrtg1 \), and lysates of cells cultured in YNB Glu + medium were subjected to Western blotting. All the proteins were expressed at significantly lower levels in \( \Delta prrtg1 \) than in GS115 (Fig. 5A). Among these PpRtg1p-regulated enzymes, PEPCK and GDH2 are essential for growth of cells in YNB Glu + medium (Fig. 5C), suggesting that deficiency of these enzymes may contribute to the growth defect of \( \Delta prrtg1 \) in YNB Glu + medium. Subcellular localization studies using PpRtg1p MYC or PpRtg1p GFP indicate that Rtg1p localizes to the cytosol in cells cultured in YNB Glu + medium (Fig. 5D and E), suggesting that PpRtg1p is unlikely to function as a nuclear transcription factor. Furthermore, mRNA levels of GDH2 and PEPCK in \( \Delta prrtg1 \) were higher but not lower than those in GS115 as evident from RT-PCR and qPCR analyses.
A promoter is widely used for recombinant protein production. Acetate, and oleic acid, and PpRtg1p is essential for the utilization of methanol, ethanol, and fatty acids as the sole source of carbon. To date, post-transcriptional regulation of AOXI has not been reported.

Table 2: Growth of P. pastoris in YNB medium containing different amino acids as the sole source of carbon and nitrogen

| Amino acids | 0 h | 12 h | 48 h |
|-------------|-----|------|------|
| Glutamic acid | 0.12 | 0.761 | 2.868 |
| Aspartic acid | 0.12 | 0.141 | 0.840 |
| Proline | 0.12 | 0.355 | 0.730 |
| Serine | 0.12 | 0.164 | 0.250 |
| Arginine | 0.12 | 0.282 | 0.211 |
| Lysine | 0.12 | 0.138 | 0.130 |
| Glycine | 0.12 | 0.134 | 0.111 |
| Threonine | 0.12 | 0.121 | 0.095 |
| Histidine | 0.12 | 0.132 | 0.090 |
| Phenylalanine | 0.12 | 0.101 | 0.073 |
| Methionine | 0.12 | 0.111 | 0.086 |
| Isoleucine | 0.12 | 0.091 | 0.067 |
| Valine | 0.12 | 0.119 | 0.066 |
| Cysteine | 0.12 | 0.068 | 0.058 |

(Fig. 5, F and G), indicating that PpRtg1p regulates GDH2 and PEPCk synthesis at the post-transcriptional level.

PpRtg1p is essential for the utilization of methanol, ethanol, acetate, and oleic acid

AOX is a methanol-inducible, peroxisomal enzyme whose promoter is widely used for recombinant protein production (12, 13). AOX is encoded by AOXI and AOXII of which AOXI accounts for >90% of AOX protein synthesized during methanol metabolism (12, 13). AOX is essential for the utilization of methanol and fatty acids as the sole source of carbon. To date, post-transcriptional regulation of AOXI has not been reported. Methanol-inducible expression of AOXI is mediated by the zinc finger proteins Mxr1p and Trm1p at the transcriptional level. AOXI mRNA as well as protein levels are down-regulated in Δmrx1 and Δtrm1 cells cultured in YNBM medium (19–21).

The down-regulation of AOXIMyc in Δprr1p1 cultured in YNB Glu+ medium (Fig. 5B) led us to investigate whether PpRtg1p regulates AOXI expression during methanol metabolism. The results indicate that AOXIMyc protein levels are significantly lower in Δprr1p1 than those in GS115 cultured in YNBM (Fig. 6, A and B). The differential expression of AOXI in GS115 and Δprr1p1 can be visualized in Coomassie Brilliant Blue–stained SDS-polyacrylamide gels because it is the most abundant protein in P. pastoris cells cultured in YNBM medium (Fig. 6C). These results were further confirmed by Western blotting of lysates of cells cultured in YNBM medium using anti-AOX antibodies, and Alexa Flour 555–conjugated donkey anti-mouse antibodies were used. Hoechst 33342 was used to stain the nucleus.

Figure 3. Analysis of the function and subcellular localization of ScRtg1p and PpRtg1p in P. pastoris cultured in YNBD medium. A, confirmation of the absence of RTG1 mRNA in Δprr1p1 by RT-PCR. PpRTG1- and PGK-specific primers were used for amplification of PpRTG1 and PGK, respectively. DNA molecular weight markers (kb) are shown on the left. B, analysis of growth of GS115 and Δprr1p1 in glutamate-deficient and -sufficient YNBD media. C, schematic diagrams of constructs designed to express PpRtg1p and ScRtg1p in Δprr1p1 as GFP fusion and 3XFLAG-tagged proteins, respectively, from PpRTG1 promoter. Numbers in parentheses indicate molecular mass of proteins (kDa). D, analysis of expression of PpRtg1pGFP and ScRtg1p3FLAG by Western blotting using anti-GFP and anti-FLAG antibodies, respectively. Numbers indicate protein molecular mass markers (kDa). E, localization of ScRtg1pFlag and PpRtg1pGFP using anti-FLAG and anti-GFP antibodies, respectively, in P. pastoris strains cultured in YNBD Glu+ medium as indicated. Mouse anti-GFP antibodies, mouse anti-FLAG antibodies, and Alexa Flour 555–conjugated donkey anti-mouse antibodies were used. Hoechst 33342 was used to stain the nucleus.

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| Threonine | 0.12 | 0.121 | 0.095 |
| Histidine | 0.12 | 0.132 | 0.090 |
| Phenylalanine | 0.12 | 0.101 | 0.073 |
| Methionine | 0.12 | 0.111 | 0.086 |
| Isoleucine | 0.12 | 0.091 | 0.067 |
| Valine | 0.12 | 0.119 | 0.066 |
| Cysteine | 0.12 | 0.068 | 0.058 |

(Fig. 5, F and G), indicating that PpRtg1p regulates GDH2 and PEPCk synthesis at the post-transcriptional level.
Post-transcriptional regulation by Rtg1p

YNBE, YNBA, and YNBO but not YNBD and YNBG (Fig. 7E). PpRtg1p predominantly localized to the cytosol irrespective of the carbon source used for culturing the cells (Fig. 7F). Thus, PpRtg1p is a cytosolic protein that regulates multiple metabolic pathways in P. pastoris while retaining its ability to act as a nuclear, retrograde transcription factor in S. cerevisiae (Fig. 8).

Discussion

S. cerevisiae, a Crabtree-positive yeast with a respirofermentative metabolism, utilizes the TCA cycle primarily as a source of α-ketoglutarate and other biosynthetic precursors while generating ATP through glycolysis (1, 2). In cells with an impaired TCA cycle, the RTG pathway provides a means of ammonium assimilation and a source of glutamate. In contrast, Crabtree-negative yeasts with a respiratory metabolism such as P. pastoris utilize the TCA cycle not only for the generation of biosynthetic intermediates but also for ATP generation via oxidative phosphorylation. Thus, mitochondrial dysfunction in these yeasts results in the deficiency of glutamate as well as ATP, and restoration of glutamate levels alone via the Rtg1p/Rtg3p-mediated retrograde pathway does not appear to be a viable option. Thus, the necessity for glutamate biosynthesis via the RTG pathway in these yeasts is questionable. The fact that deletion of RTG1 does not result in glutamate auxotrophy in P. pastoris led us to examine the function of PpRtg1p. We demonstrate that Rtg3p is absent in P. pastoris and that PpRtg1p has acquired a novel function as a cytosolic regulator of multiple metabolic pathways in P. pastoris while retaining its ability to function as a nuclear, retrograde transcription factor in S. cerevisiae.

Interestingly, ScRtg1p does not function as a cytosolic regulator in P. pastoris, indicating that PpRtg1p is a functional homologue of S. cerevisiae Rtg1p, but the latter is not a functional homologue of PpRtg1p. Although this study focused primarily on PpRtg1p, it will be interesting to examine the localization and function of Rtg1p of these yeasts.

In this study, we demonstrate that PpRtg1p regulates multiple metabolic pathways. Of these, we have identified the targets of PpRtg1p in at least two metabolic pathways. PpRtg1p regulates the synthesis of PEPCK, GDH2, GUT1, and AOX1 during glutamate utilization, whereas AOX1 is a target during methanol utilization. Proteins regulated by PpRtg1p during ethanol, acetate, and oleic acid metabolism remain to be identified. PpRtg1p localizes to the cytosol and down-regulates protein but not mRNA levels of PEPCK and GDH2 during glutamate...
utilization. During methanol metabolism, in addition to a decrease in AOX protein, AOXI mRNA levels are also affected in Δprtg1 cultured in YNBM. The exact mechanism by which PpRtg1p regulates glutamate and methanol metabolism remains to be investigated. This study demonstrates, for the first time, regulation of methanol-inducible expression of AOXI by a cytosolic protein in P. pastoris. To date, strategies aimed at manipulating the transcriptional regulatory circuits (14, 23–27) have focused on manipulating the transcriptional regulatory circuit could lead to the development of novel synthetic biology tools for genetic manipulation of recombination plasmids and expression of recombinant proteins, respectively. Bacterial and yeast transformations were done using CaCl₂ and an electroporation method (Gene Pulser, Bio-Rad), respectively, according to the manufacturer’s instructions.

Experimental procedures

Media and culture conditions

S. cerevisiae BY4741 and Δrtg1 strains were obtained from Euroscarf, Frankfurt, Germany. P. pastoris GS115 was a kind gift from James Cregg and has been described (19). P. pastoris KM71 strain was purchased from Thermo Fisher Scientific. Yeast strains were cultured at 30 °C in an orbital shaker at 180 rpm overnight in YPD (1.0% yeast extract, 2.0% peptone, 2.0% glucose), washed with sterile water, and shifted to different minimal media containing 0.17% YNB without amino acids and with 0.5% ammonium sulfate supplemented with 2.0% glucose (YNBD), 2.0% glycerol (YNBG), 1.0% glutamate (YNB Glu⁻), 1.0% methanol (YNBM), 1.0% ethanol (YNBE), or 0.5% oleic acid (YNBO). For solid medium, agar was added to a final concentration of 2%. In some experiments, YNB medium without ammonium sulfate (YNB⁺) was used. For the growth of S. cerevisiae BY4741 strain, YNB medium was supplemented with histidine (1.8 mg/ml), leucine (38 mg/ml), methionine (7.6 mg/ml), and uracil (7.6 mg/ml). Where indicated, glutamate was added at a final concentration of 0.2% to YNBD medium. For growth kinetics, colonies were first cultured overnight in YPD, then washed with sterile water, and shifted to different media with initial A₆₀₀ of ~0.1 per ml of media. Aliquots of cells were removed at regular intervals, and A₆₀₀ was measured. For spot assays, colonies were first cultured overnight in YPD, washed, and resuspended in sterile water to an A₆₀₀ of 1 per ml. Serial dilutions of 1:10 until an A₆₀₀ of 10⁻⁴ per ml were made, and 2 μl from each dilution was then spotted on solid medium.

E. coli DH5α and BL21 (DE3) strains were used for the isolation of recombinant plasmids and expression of recombinant proteins, respectively. Bacterial and yeast transformations were done using CaCl₂ and an electroporation method (Gene Pulser, Bio-Rad), respectively, according to the manufacturer’s instructions.
Antibodies and other reagents

Oligonucleotides were purchased from Sigma-Aldrich. Anti-FLAG tag and anti-Myc tag antibodies were purchased from Sigma-Aldrich and Merck Millipore, respectively. Mouse anti-GFP and mouse anti-His tag antibodies were purchased from Santa Cruz Biotechnology Inc. Donkey anti-mouse Alexa Flour 555/488 antibodies were purchased from Thermo Fisher Scientific. Anti-AOXI antibodies were generated by injecting AOXI purified from *P. pastoris* cell extracts into mice. Anti-phosphoglycerate kinase (PGK) antibodies were obtained by immunizing rabbits with recombinant, histidine-tagged *P. pastoris* PGK. Nucleotide sequence of primers used in qPCRs and RT-PCRs will be provided on request. Restriction enzymes, *Tag* DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Frankfurt, Germany).

qPCR and RT-PCR

Total RNA was isolated from yeast cells using an RNA isolation kit (Promega), and qPCR was carried out essentially as described (21) using a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). For semiquantitative RT-PCR, cDNA was prepared using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and PCR was carried out for 20, 25, and 30 cycles in a 7270 thermal cycler (Thermo Fisher). PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining (0.05 μg/ml) using a UV transilluminator.

Subcellular localization studies

Yeast cells were treated with 1% formaldehyde for 1 h and resuspended in buffer containing 40 mM K$_2$HPO$_4$ (pH 7.4), 1.2 M sorbitol, 50 mM MgCl$_2$, and 30 mM β-mercaptoethanol. Zymolyase (G Biosciences, St. Louis, MO) was added (7 μl/100 μl) and incubated at 37 °C for 1 h. Cells were centrifuged at 1500 rpm for 10 min in a microcentrifuge (Hettich, Tuttingen, Germany) at room temperature, resuspended in phosphate-buffered saline (PBS), spread evenly onto glass coverslips, and then air-dried. Coverslips were incubated in blocking buffer (PBS containing 0.05% Tween 20 and 2% BSA) for 1 h followed by incubation in blocking buffer containing primary and secondary antibodies at room temperature for 1–3 h. Coverslips were washed with PBS and then treated with Hoechst 33342 (Sigma-Aldrich; 1 μg/ml) or 4’,6-diamidino-2-phenylindole (DAPI) (1 μg/ml) for 7 min. After washing with PBS, coverslips were air-dried, and cells were visualized using a fluorescence microscope (Leica DMLA) or confocal microscope (Zeiss LSM 880 with Airyscan) with appropriate filters.

Figure 6. Regulation of AOX expression by PpRtg1p during methanol metabolism of *P. pastoris*. A, analysis of AOX Myc levels in GS115 and ΔPprtg1 cultured in YNBM medium by Western blot analysis using anti-Myc epitope antibodies. Two independent clones of GS115 (1 and 2) and ΔPprtg1 (3 and 4) were examined. PGK served as a loading control. Protein molecular mass markers (kDa) are indicated. B, quantification of data in A. Error bars represent ± S.D. of two independent biological replicates. C, direct visualization of AOX protein levels in the lysates of GS115 and ΔPprtg1 cultured in YNBM medium by SDS-PAGE and Coomassie Brilliant Blue staining. Cells were cultured in YNBM for different durations as indicated. D, confirmation of differential expression of AOX in GS115 and ΔPprtg1 cultured in YNBM medium by Western blotting using anti-AOX antibodies. PGK served as a loading control. Protein molecular mass markers (kDa) are indicated. Quantification of data in D is also shown. Error bars indicate mean ± S.D. (n = 3). *** p < 0.0005. E, analysis of differential expression of peroxisomal AOX in GS115 and ΔPprtg1 cultured in YNBM medium by immunofluorescence using mouse anti-AOX antibodies and Alexa Flour 555–conjugated donkey anti-mouse antibodies. The nucleus was stained with Hoechst 33342. F, analysis of relative levels of AOX protein in GS115, ΔPprtg1, and Δmxr1 by semiquantitative RT-PCR. M, DNA molecular weight markers (kbp). Numbers on the top indicate the number of PCR amplification cycles. H, analysis of AOX mRNA levels in GS115, ΔPprtg1, and Δmxr1 by qPCR. Error bars indicate mean ± S.D. (n = 3). *** p < 0.0005.
For visualization of GFP-tagged proteins by live-cell imaging, yeast cells were placed on a glass slide layered with 1% agarose, and the cells were allowed to settle for 1 min. A coverslip was placed on top of the cell suspension, the edge of the coverslip was sealed with nail polish, and cells were visualized using a confocal microscope as mentioned above.

EMSA

EMSA was carried out essentially as described (20). Briefly, oligonucleotides radiolabeled at their 5'-ends using [γ-32P]ATP and T4 polynucleotide kinase were incubated with recombinant proteins in EMSA buffer (50 mM Tris (pH 8.0), 250 mM NaCl, 0.25% Nonidet P-40, 5 mM DTT, and 30% glycerol). The reaction mixture was electrophoresed on a 5% polyacrylamide gel at 4 °C for 6 h. The gel was dried and exposed to a phosphorimaging screen overnight. DNA and DNA–protein complexes were visualized using a Typhoon FLA 9000 laser scanner.

Western blotting

Yeast cells were resuspended in a lysis buffer containing 20 mM Tris (pH 8.0), 400 mM NaCl, 10 mM MgCl2, 10 mM EDTA (pH 8.0), 10% glycerol, 7 mM β-mercaptoethanol, and protease inhibitor mixture (cOmplete, ULTRA, Mini, EDTA-free, EASYpack). Chilled, 0.5-mm glass beads (Biospec Products) were added (1 g/g of cells) and vortexed 10 times for 1 min, each time keeping the cells on ice for 1 min between vortexings and using the highest setting of the vortex mixer. Cell debris was removed by centrifugation at 13,000 rpm in a microcentrifuge (Hettich). Proteins were estimated using Bradford reagent (Bio-Rad) and resolved by SDS-PAGE, electroblotted onto a 0.22-μm polyvinylidene difluoride membrane using transfer buffer (39 mM glycine, 48 mM Tris (pH 8.0), and 20% methanol). The membrane was blocked overnight in 5% nonfat milk.
Post-transcriptional regulation by Rtg1p

(HiMedia, Mumbai, India) prepared in TTBS (25 mM Tris (pH 8.0), 0.1% Tween 20, and 125 mM NaCl). Blots were sequentially incubated in TTBS containing antibodies raised against a specific protein or anti-epitope tag antibodies of appropriate dilution for 1–3 h. Primary antibodies were detected by peroxidase-conjugated anti-rabbit/anti-mouse IgG (1:10,000 dilution). Proteins were detected using Immobilon Western Chemiluminescent HRP substrate (Millipore) according to the manufacturer’s instructions.

Statistical analysis

Statistical tests, including Student’s t test and one-way analysis of variance followed by Tukey’s multiple comparison test, were carried out using GraphPad Prism 5 software. Data are presented as mean ± S.D. A p value summary is indicated on the bar of each figure: *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ns, not significant.

Mass spectrometry

Protein bands of interest were excised precisely from the SDS-polyacrylamide gel and subjected to in-gel trypsin digestion using a Trypsin Profile IGD kit (Sigma-Aldrich). MALDI-TOF was performed in an HCT Ultra PTM Discovery System (ETD II, Bruker Daltonics) with an 1100 series HPLC (Agilent). For identification of proteins, the “peak list” was searched against the NCBI nonredundant database using Mascot protein mass fingerprint software.

Expression of recombinant S. cerevisiae and P. pastoris Rtg proteins in E. coli and study of protein–protein interactions

For the construction of pGEX-PpRTG1 plasmid, PpRTG1 gene was amplified from GS115 genomic DNA by PCR using the primer pair 5'-CGCGGATCCATGGAATTCG-3' and 5'-AAAACTGCAGTTAGTTCGGA-3' (BamHI and XhoI sites are underlined). The PCR product was digested with BamHI and PsI sites and cloned into pMAL-c2X vector. pMAL-PpRTGX expression plasmid, expressing a chimera of PpRTGX and ScRtg3, was constructed by amplifying PpRTGX gene from GS115 genomic DNA and ScRtg3 gene encoding amino acids 345–486 from S. cerevisiae BY4741 by PCR using the following primer pairs: 5'-CGTGCTTTGATTTTCTCTTTTG-3' and 5’-CAGGGGACCG-3'. Both PCR products were purified and used as templates in the final PCR using primer pair 5’-CGCGGATCCATGGAATTCGTGCTTGTA-3’ and 5’-AAAACTGCAGTTAGTTCGGAA-3’ respectively. Both PCR products were digested with BamHI and cloned into pMAL-c2X vector.

Recombinant plasmids were transformed into E. coli BL21 (DE3) strain. Recombinant proteins were expressed as MBP fusion proteins (PpRtgXp, PpRtgXp*, and ScRtg3p) or GST fusion proteins (PpRTG1 and ScRTG1) and purified using amylose resin (New England Biolabs) and GSH-agarose beads (G Biosciences), respectively, according to the manufacturers’ instructions.

For the study of protein–protein interactions, E. coli cells were suspended in a buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 10% glycerol, 10 mM β-Mercaptoethanol, and 1 mM PMSF and subjected to sonication in a Vibracell 750 (Sonics and Materials Inc., Newtown, CT). Cell lysates containing GST-Rtg1p were incubated with GSH-agarose beads at 4 °C for 1 h followed by brief centrifugation and washing with a buffer A containing 50 mM Tris (pH 8.0) and 150 mM NaCl. GST-Rtg1p bound to GSH-agarose beads was incubated with E. coli cell lysates containing MBP-Rtg3p, MBP-RtgXp, or MBP-RtgXp* for 1 h. After centrifugation and washing twice with buffer A, proteins bound to GSH beads were resolved on an SDS-polyacrylamide gel and visualized by Coomassie Brilliant Blue R staining.

Generation of S. cerevisiae ∆Sc-ScRTG1-GFP and ∆Sc-PpRTGX-GFP

ScRTG1 along with ~1.0-kb promoter was amplified by PCR from S. cerevisiae BY4741 genomic DNA using the primer pair 5’-CGCGGATCCATGGAATTCGTGCTTGTA-3’ and 5’-CTCCTTTACTACTCTACAGCTCACCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCACCCATTAGTCACCATTAGTCAAGG-3' (BamHI and PsI restriction sites are underlined). The PCR product was digested with BamHI and cloned into pMAL-c2X vector.

PpRTGX gene was amplified from GS115 genomic DNA using the primer pair 5’-GGAACCAAAAGAAAGCACAAGGCA-3’ and 5’-AAGGAAAAAAGCGGCCGCTCAATCCATGCTATGCTACCATTA-3' (BamHI and XhoI sites are underlined). The PCR product was digested with BamHI and PsI restriction sites and cloned into pMAL-c2X vector.
TCAACACCAATG-3'. ScRTG1 promoter was separately amplified from S. cerevisiae BY4741 genomic DNA using primer pair 5’-CCGGATCCGTACTGAGGTGAATCCATC-3’ and 5’-CTTGGCGCTATGTTGGCTATTG-3’. The gene encoding GFP was amplified from the pREP41GFP vector (28) using primer pair 5’-CATATGGTATGCTGGATGGAGCG-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’. The three individual PCR products were purified and used as templates in a final PCR with the primers containing BamHI and XhoI sites (XhoI site is underlined) to generate SmacASScRTG1-GFP and SmacASScRTG1FLAG downstream of ScRTG1 promoter.

**Generation of P. pastoris ΔPtrtg1 strain**

The ΔPtrtg1 strain in which the coding region of RTG1 was replaced by a Zeocin expression cassette was generated as follows. The 1.08-kb RTG1 promoter was amplified from P. pastoris GS115 genomic DNA by PCR using the primer pair 5’-GATTTCGTGAGGATCCATGTTCCATC-3’ and 5’-CTATTGACCATCGGATCCACACACAGATG-GTGAGATCTGAGAAGG-3’ (962 to 984 bp in reverse complement) (in italics), −43 to −20 bp of RTG1 in reverse complement. In the second PCR, 1.2 kb of the Zeocin expression cassette was amplified by PCR using the primer pair 5’-CTTCACTACTCTGTGACTCATGCGGATCCACACACAGATG-GTGAGATCTGAGAAGG-3’ (−43 to −20 bp of RTG1 (italics), +962 to +984 bp of pGAPZA vector) and 5’-GAGAAGAATCTGATCAGAGATCACTTCACATGGTGTGCTCCAGATTGTTG-3’ (+743 to +767 bp in reverse complement) of 3’-flanking region of RTG1 (in italics), +2137 to +2159 bp in reverse complement of pGAPZA. In the third PCR, 974 bp of 3’-flanking region of RTG1 was amplified using the primer pair 5’-CAAGCTTAGAAACATGCTGAGGATCCACACACAGATG-GTGAGATCTGAGAAGG-3’ and 5’-ATACTGCATATGAGGTTCATCCACACACAGATG-GTGAGATCTGAGAAGG-3’ (+1693 to +1716 bp in the reverse complement of 3’-flanking region of RTG1). All three PCR products were purified and used as templates in the final PCR along with the primer pair 5’-GATTTCGTGAGGATCCATGTTCCATC-3’ and 5’-ATACATCCTCTGCTCATGTTCCACACACAGATG-GTGAGATCTGAGAAGG-3’ to obtain a 3.47-kb product consisting of the Zeocin expression cassette flanked by 1.080 kb of RTG1 promoter and 0.974 bp of 3’-flanking region of RTG1 that was transformed into P. pastoris GS115 strain. Zeocin-resistant transformants that do not express RTG1 mRNA as evident from RT-PCR were selected and designated as ΔPtrtg1.

**Generation of P. pastoris ΔPP-Ptrtg1**

Expression cassettes comprising genes encoding PpPtrg1 and ScRtg1 along with ~1 kb of PpRTG1 promoter were cloned into pIB3 vector (Addgene, 25452) and expressed in ΔPtrtg1 as GFP-tagged and FLAG-tagged proteins, respectively. PpRTG1 (1.741 kb) along with its ~1-kb promoter was amplified from GS115 genomic DNA using the primer pair 5’-CCGGCTCGAGGATTTCTCGAGGAGG-3’ and 5’-CTCTTATCTGACATCTCATCATCATCTGATGTTGTTCCCATC-3’ (XhoI site is underlined), and a 741-bp coding region of GFP was amplified from the vector pREP41GFP vector (28) using the primer pair 5’-CATATTGATTGGACCACTAGGACATTGAGATCTGACTGTTGTTCCCATC-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’. The PCR product was cloned into pIB3 vector and transformed into E. coli DH5α competent cells. Recombinant plasmid containing Pp-ScRTG1FLAG construct was linearized using Sall and transformed into ΔPtrtg1 by electroporation. Recombinant clones were selected by plating on YNB His− plates, and clones expressing GFP-tagged Rtg1p were confirmed by Western blotting using anti-GFP antibody.

For the generation of ΔPP-ScRTG1FLAG, ~1-kb PpRTG1 promoter, ScRTG1 gene, and FLAG tag were amplified from genomic DNA isolated from GS115, BY4741, and Mxrt1FLAG strains (29), respectively, using overlapping primer pairs 5’-CCGCTCGAGGATTTCTCGAGGAGG-3’ and 5’-GCCACGTGGAATGCTGCTCATGAGGATGTCCTGGCCAGCTG-3’, 5’-GACCTCCATCTGACAGCATTCCATGGAATGCTGCTCATGAGGATGTCCTGGCCAGCTG-3’, and 5’-GACCTCCATCTGACAGCATTCCATGGAATGCTGCTCATGAGGATGTCCTGGCCAGCTG-3’. The PCR products were purified and used as templates in the final PCR along with the primer pair 5’-CCGCTCGAGGATTTCTCGAGGAGG-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’ (XhoI site is underlined) to obtain a 3.234-kb product consisting of the 1-kb promoter was amplified from the vector pREP41GFP vector (28) using the primer pair 5’-CATATTGATTGGACCACTAGGACATTGAGATCTGACTGTTGTTCCCATC-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’. The PCR product was cloned into pIB3 vector and transformed into E. coli DH5α competent cells. Recombinant plasmid containing PpRTG1-ScRTG1FLAG construct was linearized using Sall and transformed into ΔPtrtg1 by electroporation. Recombinant clones were selected by plating on YNB His− plates, and clones expressing GFP-tagged ScRtg1p were confirmed by Western blotting using anti-FLAG antibody.

**Generation of GS-PP-RTG1Myc**

P. pastoris GS115 strain expressing Myc epitope–tagged PpPtrg1p (GS-PP-RTG1Myc) was generated by cloning PpPtrg1p along with ~1 kb of its promoter as a TAP-tagged protein (30), which also contains a Myc epitope. PpRTG1 (1.741 kb) along with its promoter was amplified from GS115 genomic DNA using the primer pair 5’-CCGCTCGAGGATTTCTCGAGGAGG-3’ and 5’-GAAATTTTCTTTCTACATCGTACGTTGTTCCCATC-3’ (XhoI site is underlined). TAP was cloned from pYM13 vector (Euroscarf) using primer pair 5’-GTTTGGGACCACTAGCATGAGAAAGAAGATGGGAGAAAGGACTTCCATC-3’ and 5’-ATAAGAATGCGGCCGCTTAAAGGATCC-3’ (NotI site is underlined). Both PCR products were purified and used as a templates in the final PCR along with the primer pair 5’-CCGCTCGAGGAGG-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’ (NotI site is underlined). Both PCR products were purified and used as a templates in the final PCR along with the primer pair 5’-CCGCTCGAGGAGG-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’ (NotI site is underlined). Both PCR products were purified and used as a templates in the final PCR along with the primer pair 5’-CCGCTCGAGGAGG-3’ and 5’-CCCAAGCTTCTAGTGGGTTTGTGGC-3’ (NotI site is underlined).
Post-transcriptional regulation by Rtg1p

The primer pair 5′-ATAAGATGGCGCCGCGCC-3′ and 5′-ATAAGATGGCTGAGGACGATG-3′ was amplified from genomic DNA using PCR. The PCR product was cloned into pGAPZA vector (29) and transformed into E. coli DH5α competent cells. Recombinant plasmid containing RTG1-TAP construct was linearized using AvrII and transformed into GS115 by electroporation. Recombinants were selected on blasticidin-containing YPD plates. Recombinants expressing TAP-tagged PpRtg1p were selected by Western blotting using anti-Myc antibody.

Generation of GS-PpRTG1GFP-OE

P. pastoris GS115 expressing PpRtg1p as a GFP fusion protein from GAPDH promoter (GS-PpRTG1GFP-OE) was generated as follows. PpRtg1 gene (741 bp) was amplified from GS115 genomic DNA using primers 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ and 5′-CCCAGCTTCAGATGATGCTGAGGACGATG-3′ (XhoI site is underlined, in reverse complement) and cloned into pGAPZA vector. The PCR product was cloned into E. coli DH5α competent cells. Recombinant plasmid containing GAPZA_PpRTG1-GFP-OE was linearized with AvrII and transformed into GS115 by electroporation. Recombinants were selected on Zeocin expression cassette flanked by 1 kb of genomic DNA using PCR. To generate the GS-PpRTG1GFP-OE construct, PpRtg1 gene was replaced by a zeocin expression cassette was amplified by PCR using the primer pair 5′-GCTGGAGGAAAATCTTATGAGGACGATG-3′ and 5′-CCAGCTTCAGATGATGCTGAGGACGATG-3′ (XhoI and HindIII sites are underlined). The PCR product was cloned into pGAPZA vector using overlapping primers. First, PpRtg1 promoter (1.0 kb) was amplified from P. pastoris genomic DNA isolated from GS115 and BY4741, respectively, using overlapping primer pairs 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ and 5′-GCGAGCTTATGAGGACGATG-3′. ScRTG1 gene was amplified from S. cerevisiae genomic DNA using the primer pair 5′-GACTCCATCTAAGAAGACTATCTTATGAGGACGATG-3′ and 5′-CCCATGCTGAGTATGATGCTGAGGACGATG-3′. Both PCR products were purified and used as templates in the final PCR along with the primer pair 5′-CCGCTCGAGATTCATGATGCTGAGGACGATG-3′ and 5′-CCGAGGTTCAATCTTATGATGCTGAGGACGATG-3′ (XhoI and HindIII sites are underlined). The PCR product was linearized using Sall and transformed into ΔPpRtg1 strain by electroporation. Recombinant clones were selected by plating on YNBD His− plates, and expression of ScRTG1 in the recombinant clones was confirmed by RT-PCR.

Generation of P. pastoris GS-ΔPEPCK and GS-ΔGUT1 strains

P. pastoris ΔPEPCK strain in which the coding region of PEPCK was replaced by a zeocin expression cassette was generated as follows. PEPCK promoter (−1000 bp) was amplified by PCR using the primer pair 5′-CTACATCGGAAAACATTGAGGACGATG-3′ and 5′-CTATGCTGAGTATGATGCTGAGGACGATG-3′ (−1000 to −971 bp of PEPCK) and 5′-CAATTCCAGGATGATGCTGAGGACGATG-3′ and 5′-GAAATATATTATTTG-3′ (−971 to −947 bp of PEPCK). In the second PCR, 1.2 kb of zeocin expression cassette was amplified by PCR using the primer pair 5′-GGAATATATTATTTGAGGACGATG-3′ and 5′-GCTGGAGGAAAATCTTATGAGGACGATG-3′ (−947 to +1698 bp in reverse complement of 3′ flanking region of PEPCK [in italics], +2137 to +2159 bp in reverse complement of PEPCK). In the third PCR, 1 kb of the 3′ flanking region of PEPCK was amplified using the primer pair 5′-GCTGGAGGAAAATCTTATGAGGACGATG-3′ and 5′-GAAATATATTATTTGAGGACGATG-3′ (−2159 to +2159 bp of PEPZA [in italics], +2176 to +2198 bp of PEPCK in reverse complement). All three PCR products were purified and used as templates in the final PCR along with the primer pair 5′-CTACATCGGAAAACATTGAGGACGATG-3′ and 5′-CAATTCCAGGATGATGCTGAGGACGATG-3′ to obtain an ~3.2-kb product consisting of Zeocin expression cassette flanked by 1 kb of PEPCK promoter and 1 kb of 3′-flanking region of PEPCK that was transformed into P. pastoris GS115 strain. Zeocin-resistant colonies were selected, and deletion of PEPCK was confirmed by the absence of PEPCK gene in the genomic DNA using PCR.

P. pastoris GS-ΔGUT1 strain in which 1 kb from the 1.8-kb GUT1 coding region was replaced by a zeocin expression cassette was generated as follows. First, ~1 kb of GUT1 promoter was amplified by PCR using the primer pair 5′-CGCTCGGAGGAAAATCTTATGAGGACGATG-3′ and 5′-CTATGCTGAGTATGATGCTGAGGACGATG-3′ (−973 to −947 bp of GUT1 with XhoI site (underlined)) and 5′-CTATGCTGAGTATGATGCTGAGGACGATG-3′ (962 to 985 bp of PEPZA in reverse complement (in italics), −25 to −1 bp of GUT1 in reverse complement). The 1.2-kb Zeocin expression cassette was amplified by PCR using the primer pair 5′-GCG-3′ and 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ (−24 to −1 bp of PEPCK in reverse complement). In the second PCR, 1.2 kb of zeocin expression cassette was amplified by PCR using the primer pair 5′-GCG-3′ and 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ (−1675 to +1698 bp in reverse complement of 3′ flanking region of GUT1 [in italics], +2137 to +2159 bp in reverse complement of GUT1). In the third PCR, ~1 kb of the 3′ flanking region of GUT1 was amplified using the primer pair 5′-GCG-3′ and 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ (−24 to −1 bp of GUT1 in reverse complement). In the third PCR, ~1 kb of the 3′ flanking region of GUT1 was amplified using the primer pair 5′-GCG-3′ and 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ (−1675 to +1698 bp in reverse complement of 3′ flanking region of GUT1). All three PCR products were purified and used as templates in the final PCR along with the primer pair 5′-GCG-3′ and 5′-CCGCTCGAGATGGATGATGCTGAGGACGATG-3′ to obtain an ~3.2-kb product consisting of Zeocin expression cassette flanked by 1 kb of PEPCK promoter and 1 kb of 3′-flanking region of PEPCK that was transformed into P. pastoris GS115 strain. Zeocin-resistant colonies were selected, and deletion of PEPCK was confirmed by the absence of PEPCK gene in the genomic DNA using PCR.
the primer pair 5’-CTATACCCAGATATACTCATATATGCAGATCCCCACACACCAGATG-3’ (−25 to −1 bp of GUT1 promoter (in italics), +962 to +985 bp of pGAPZA) and 5’-CTGTCACAAACAAGAGGACATGTTGCTCAGATGGTGGTGCTCCAGTTGC-3’ (+966 to +991 bp in reverse complement of 3’-coding region of GUT1) (+1231 to +2131 bp in reverse complement of pGAPZA). In the third PCR, −880 bp of the 3’-coding region of GUT1 was amplified using the primer pair 5’-CGGGAATTCCTCTCATGTTCGGAACCTAAGACCGGTCTTCTCAG-3’ (+2131 to +2159 bp of pGAPZA) (in italics), +966 to +991 bp of coding region of GUT1) and 5’-CCCAAGCTTTCAGTGTCCTCTTAAAGC-3’ (+1882 to +1897 bp in the reverse complement of 3’-coding region of GUT1 with HindIII site (underlined)). All three PCR products were purified and used as templates in the final PCR along with the primer pair 5’-CGGGAATTCCTCTCATGTTCGGAACCTAAGACCGGTCTTCTCAG-3’ and 5’-CCCAAGCTTTCAGTGTCCTCTTAAAGC-3’ to obtain an 13-kb product consisting of Zeocin expression cassette flanked by 1 kb of GUT1 promoter and ~800 bp of 3’-flanking region of GUT1 that was transformed into GS115. Zeocin-resistant colonies were selected, and deletion of GUT1 was confirmed by the absence of 1 kb of GUT1 gene in genomic DNA using PCR.

**Table 3**

List of yeast strains used in this study

For the sake of brevity, GS115, ∆prr1g, and ∆scrtg1 are referred to as GS, Δp, and Δs, respectively.

| Yeast strain | Genotype |
|--------------|----------|
| S. cerevisiae | BY4741a Scrtg1 | S288C (MATa his3-D0 leu2-D0 met15+0 ura3-D0) Euroscarf |
|             | BY4741 ⋆Scrtg1 | BY4741 carrying Scrtg1 deletion Euroscarf |

| P. pastoris | Source/Ref. |
|-------------|-------------|
| GS115       | thermo Fisher |
| KM71        | thermo Fisher |
| Δprr1g      | thermo Fisher |
| Δp-PpRTG1OGP | thermo Fisher |
| Δp-PpRTG1c  | thermo Fisher |
| Δp-PpRTG1a  | thermo Fisher |
| Δp-PpRTG1OE | thermo Fisher |
| GS-GDH2His | thermo Fisher |
| GS-PpRTG1OE | thermo Fisher |
| GS-GUT1Myc | thermo Fisher |
| GS-PPEPCKMyc | thermo Fisher |
| GS-AOXIMyc | thermo Fisher |
| GS-PpRTG1OE | thermo Fisher |
| GS-PEPCKMyc | thermo Fisher |
| GS-GDHB | thermo Fisher |

**Post-transcriptional regulation by Rtg1p**

See text for details.

**Generation of P. pastoris GS-GDH2His, Δp-PpGDH2His, GS-PPEPCKMyc, Δp-PpPEPCKMyc, Δp-Pp-AOXIMyc, Δp-AXOMyc, and Δp-GUT1Myc strains**

Expression cassettes comprising genes encoding PpPCK and GUT1 along with ~1 kb of their promoters were cloned into pBl3 vector (Addgene) and expressed in P. pastoris GS115 and P. pastoris Δrtg1g (Δprr1g) as Myc-tagged proteins. AOXI was cloned with ~1 kb of its promoter in pGAPZA vector as a Myc-tagged protein, whereas GDH2 was cloned with 545 bp of its promoter in pBl3 vector as a His-tagged protein. PpPCK and GUT1 were amplified from GS115 genomic DNA using primer pairs 5’-GGGTTACCCCACTGCGTCGC-3’ and 5’-AAACGGTCATGCTGCTGGTG-3’ (KpnI and HindIII sites are underlined) and 5’-CCGGTAAAGGCGCGTTCG-3’ and 5’-CCCAAGCTTCAGTGTCCTCTTAAAGC-3’ (EcoRI and XhoI sites are underlined). PCR products were cloned into pBl3 vector and transformed into E. coli DH5α competent cells. Recombinant plasmids containing PpPPEPCKMyc and PpGUT1Myc were linearized using BsrGI and Sall, respectively, and transformed into GS115 and Δprr1g by electroporation. Recombinant plasmid containing PpGUT12-ΔpGUT1-MyC was linearized using Stul and transformed by electroporation into GS115 and Δprr1g. Recombinant clones were selected by plating on YNBD His− plates, and clones expressing Myc-tagged PePCK and GUT1 and His-tagged GDH2 were confirmed by Western blotting using anti-Myc and anti-His antibodies.

AOXI was amplified from GS115 genomic DNA by the primer pair 5’-CGGGAATTCCTCTCATGTTCGGAACCTAAGACCGGTCTTCTCAG-3’ and 5’-ATATCGGAGCCTGCTGCTGGTG-3’. PCR products were cloned into respective sites of pGAPZA vector and transformed into E. coli DH5α competent cells. The recombinant plasmid was linearized with AvrII and transformed into GS115 and Δprr1g by electroporation. Recombinants expressing Myc-tagged
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AOX1 were selected by Western blotting using anti-Myc antibody. The various yeast strains used in this study are listed in Table 3.

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