Casein kinases are required for the stability of the glucose-sensing receptor Rgt2 in yeast

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In yeast, glucose induction of HXT (glucose transporter gene) expression is achieved via the Rgt2 and Snf3 glucose sensing receptor (GSR)-mediated signal transduction pathway. The membrane-associated casein kinases Yck1 and Yck2 (Ycks) are involved in this pathway, but their exact role remains unclear. Previous work suggests that the Ycks are activated by the glucose-bound GSRs and transmit the glucose signal from the plasma membrane to the nucleus. However, here we provide evidence that the Ycks are constitutively active and required for the stability of the Rgt2 receptor. Cell surface levels of Rgt2 are significantly decreased in a yck1Δyck2{ts} mutant, but this is not due to endocytosis-mediated vacuolar degradation of the receptor. Similar observations are made in an akr1Δ mutant, where the Ycks are no longer associated with the membrane, and in a sod1Δ mutant in which the kinases are unstable. Of note, in an akr1Δ mutant, both the Ycks and Rgt2 are mislocalized to the cytoplasm, where Rgt2 is stable and functions as an effective receptor for glucose signaling. We also demonstrate that Rgt2 is phosphorylated on the putative Yck consensus phosphorylation sites in its C-terminal domain (CTD) in a Yck-dependent manner and that this glucose-induced modification is critical for its stability and function. Thus, these results indicate a role for the Ycks in stabilizing Rgt2 and suggest that Rgt2 may use glucose binding as a molecular switch not to activate the Ycks but to promote Yck-dependent interaction and phosphorylation of the CTD that increases its stability.

Glucose serves as both a fuel for energy and a precursor for the biosynthesis of cellular building blocks such as amino acids, fatty acids, and nucleotides. The budding yeast S. cerevisiae has a remarkable preference for glucose, because regulation of cellular function of glucose determines the organism’s distinctive fermentative metabolism—aerobic fermentation—observed in many kinds of tumor cells. Fermentation is an energy-inefficient process, but it can proceed at a much faster rate, yielding a high glycolytic flux. The resulting accumulated glycolytic intermediates serve as anabolic precursors required for the biosynthesis of macromolecules, facilitating mass accumulation, and thus accelerating cell proliferation.

The yeast cells increase their glycolytic capacity, in part, by facilitating glucose uptake through glucose transporter (HXT) genes. This is achieved through a glucose signaling pathway that begins at the cell surface with the glucose sensing receptors (GSRs) Rgt2 and Snf3 and ends in the nucleus with the Rgt1 repressor. Rgt1 represses expression of the HXT genes in the absence of glucose by recruiting the HXT corepressors Mth1 and Std1 and the general co-repressor complex Ssn6-Tup1 to the HXT promoters in the absence of glucose. Glucose induces degradation of Mth1 and Std1, causing Ssn6-Tup1 to dissociate from Rgt1 and allowing its phosphorylation by PKA (Protein Kinase A). Phosphorylated Rgt1 is dissociated from the HXT promoters, resulting in derepression of HXT genes. Therefore, glucose-induced degradation of Mth1 and Std1 is the key event that enables induction of HXT gene expression.

Evidence showed that Mth1 and Std1 are ubiquitinated by the SCF{Gln1} ubiquitin-protein ligase complex and degraded via the 26S proteasome in response to glucose and that this occurs in a GSR-dependent manner. Many SCF substrates are phosphorylated prior to ubiquitination, and the plasma membrane-tethered casein kinases Yck1 and Yck2 (herein referred to as Ycks), the homologs of the casein kinase 1-gamma (CK1γ), were shown to be responsible for the phosphorylation of Mth1 and Std1. These results led to the view that the...
glucose-activated GSRs activate Ycks, which then catalyze phosphorylation of Mth1 and Std1, priming them for ubiquitination and subsequent degradation. In this model of the GSR pathway, the Ycks act as downstream signal transmitters of the glucose receptors. More recently, however, the GSRs have been reported to be epistatic to the Ycks, placing the kinases upstream or at the level of the receptors in the GSR pathway.

Glucose, as a signaling molecule, appears to play a key role in regulating cell surface levels of GSRs: Rgt2 is expressed in the plasma membrane when glucose is abundant, turning on glucose signaling; it is endocytosed and degraded in the vacuole in response to glucose depletion, turning off signaling. Thus, the dynamic control of the cell surface levels of the GSRs is of fundamental importance in modulating the activity of the GSR pathway in response to different levels of extracellular glucose. However, the underlying mechanisms remain largely unknown.

Here, we have explored the differences between wild-type and the glucose sensing defective mutants, yck1Δyck2Δ, akr1Δ, and sod1Δ, with respect to Rgt2 protein levels at the cell surface. Using the yeast two hybrid assays, we have assessed Rgt2 interaction with the Ycks. Finally, we have performed in vitro kinase assays to assess Yck1 activity in wild-type, rgt2Δsnf3Δ, RGT2-1, and SNF3-1 strains. Our results demonstrate a role for the Ycks in stabilizing Rgt2.

**Results**

**The Ycks are required for the stability of the Rgt2 receptor.** The Ycks are involved in the GSR-mediated glucose sensing and signaling, but their exact role remains unclear. To examine the role for the Ycks in the GSR pathway, we first assessed the cell surface levels of Rgt2 in cells lacking Yck activity (yck1Δyck2Δ) by Western blot analysis. Rgt2-HA levels are significantly lower in yck1Δyck2Δ cells grown on glucose (+) or galactose (−) as compared with wild-type cells (Fig. 1A). However, no significant difference is observed in transcriptional activity of the RGT2 promoter (measured with an RGT2-lacZ reporter), suggesting that the decreased Rgt2 protein levels in yck1Δyck2Δ cells may be not due to transcriptional repression of the RGT2 gene (Fig. 1B). This is confirmed by expressing GFP-Rgt2 from the MET25 promoter, which is not regulated by glucose. The membrane-bound GFP-Rgt2 levels in yck1Δyck2Δ cells grown on or without glucose are found to be significantly low compared to those in wild-type cells (Fig. 1C, left). The rgt2 snf3 double mutant grows poorly on glucose-containing media. This growth defect is complemented by expression of Rgt2-HA and GFP-Rgt2, suggesting that both Rgt2-HA and Rgt2-HA are functional (Fig. 1C, right).

To further explore the possibility of mislocalization of Rgt2 in a yck1Δyck2Δ mutant, Rgt2-HA was immunoprecipitated from cell lysates and analyzed by Western blotting. The results reveal two distinct forms of Rgt2-HA from wild-type cells: a major slower-migrating (upper) band, corresponding to the membrane-bound Rgt2-HA (Fig. 1D, lane 1) and a minor faster-migrating (lower) band (Fig. 1D, lane 3). By contrast, Rgt2-HA from yck1Δyck2Δ cells shows only the minor, lower band, implicating a role for the Ycks in the stability of Rgt2 at the cell surface.

However, the previous work by Snowden and Johnston showed that Rgt2 levels are not significantly different between wild-type and yck1Δyck2Δ strains. To address this discrepancy, we monitored changes of Rgt2 abundance after shifting yck1Δyck2Δ cells from 26 °C to 37 °C for various periods of time. Rgt2-HA is immunoprecipitated from the cell extracts and analyzed by Western blotting. The results show that Rgt2-HA levels are decreased by ~ 50% within 30 min and indicate that Rgt2 stability may directly correlate with Yck activity (Fig. 1E). While we do not know the exact nature of this discrepancy, we shifted yck1Δyck2Δ cells to a restrictive temperature (37 °C) before adding glucose to completely inactivate the kinases, whereas they shifted them to 30 °C. However, we find that Rgt2 is unstable in yck1Δyck2Δ cells at 30 °C (Fig. 1F).

Secondly, we examined Rgt2 levels in cells lacking Sod1, an upstream regulator of the Ycks. Previous work showed that Sod1 (Cu/Zn superoxide dismutase) interacts with and stabilizes the Ycks, and consequently, the kinases are undetectable in a sod1Δ mutant. Thus, like yck1Δyck2Δ cells, sod1Δ cells are unable to properly activate HXT1. Expectedly, we find very low levels of Rgt2-HA in sod1Δ cells, supporting the view that the Ycks may be required for the stability of Rgt2 (Fig. 1G, H).

Glucose starvation-induced Rgt2 endocytosis requires the EH-domain containing protein End3. Our results show that Rgt2 is apparently downregulated at the protein level in a yck1Δyck2Δ mutant, but this does not occur through End3-mediated endocytosis (Fig. 11, yck1Δyck2Δend3Δ). Thus, the Ycks may not be involved in this process.

**Cytoplasmic Rgt2 is stable in an akr1Δ strain where Ycks are cytoplasmic.** Akr1 is a palmitoyl transferase that tethers proteins to the plasma membrane. The Ycks are targeted to the plasma membrane through palmitoylation of the C-terminal Cys-Cys sequence by Akr1. Since the yck1Δyck2Δ strain is not viable, akr1 mutations are often used to model loss of the Ycks. As reported previously, GFP-Yck1 is found to be localized to the plasma membrane in wild-type cells and uniformly distributed throughout the cytoplasm in akr1Δ cells (Fig. 2A,B). When expressed in an akr1Δ strain, cell surface levels of Rgt2-HA are dramatically decreased (Fig. 2C), without noticeable changes in transcriptional activity of the RGT2 promoter, suggesting that the membrane-bound Rgt2 is downregulated at the protein level in akr1Δ cells (Fig. 2D).

Interestingly, Rgt2-HA from akr1Δ cells are detected in immunoprecipitates of cell lysates and its levels are comparable to those of wild-type cell lysates, suggesting that Rgt2-HA is stable in the cytoplasm (Fig. 2E). This is confirmed by confocal microscopy. GFP-Rgt2 is localized in the plasma membrane of glucose-grown wild-type cells, with visible GFP signals in the vacuole, and targeted to the vacuole when the cells were shifted from glucose to galactose (Fig. 2F,G). However, GFP-Rgt2 expressed in akr1Δ cells is not properly localized to the plasma membrane but is distributed to the cytoplasm (Fig. 2F,G). Thus, both the Ycks and Rgt2 are accumulated in the
Figure 1. The Ycs are required for the stability of Rgt2 at the cell surface. (A) Western blot analysis of Rgt2-HA protein levels in WT (LRB939) and yck1Δyck2Δ (LRB1613) cells. C-terminally HA-tagged Rgt2-HA was expressed from the \( RGT2 \) promoter (\( \text{P}_{\text{RGT2}} \)). Cells were grown in selective SC medium with 2% glucose to mid-log phase (\( \text{OD}_{600\text{nm}} = 1.2 - 1.5 \)) and equal amounts of cells were shifted to SC medium containing glucose (2%) or galactose (2%) for 1 h. The \( \text{yck1Δyck2Δ} \) cells were incubated at 37 °C for 30 min before the precultures were shifted to fresh glucose medium or galactose medium. Membrane fractions were immunoblotted with anti-HA antibody. Pgk1 was used as loading control. WT levels in \( \text{WT} \) in glucose starvation-induced endocytosis and degradation of Rgt2. Western blot analysis of Rgt2-HA protein levels in \( \text{WT} \) and \( \text{yck1Δyck2Δ} \) cells. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller Units. Values are means for at least three independent experiments. (B) \( RGT2 \)-\( \text{lacZ} \) expression in WT and \( \text{yck1Δyck2Δ} \) cells. β-Galactosidase activity was assayed in permeabilized cells and expressed in Miller Units. Values are means for at least three independent experiments. (C) Western blot analysis of GFP-Rgt2 protein levels in WT and \( \text{yck1Δyck2Δ} \) cells. GFP-Rgt2 was expressed from the \( \text{MET25} \) promoter (\( \text{P}_{\text{MET25}} \)), which is not regulated by glucose(15) (left). The growth defect of the \( \text{rgt2 snf3} \) double mutant is complemented by expression of \( \text{GFP-Rgt2} \) (right). The \( \text{rgt2Δsnf3Δ} \) mutant (MSY441) transformed with \( \text{GFP-Rgt2} \) (a) and an empty vector (b) was scored for growth on SC medium containing glucose (2%) or galactose (2%). (D) WT and \( \text{yck1Δyck2Δ} \) cells expressing Rgt2-HA were grown as described in (A). Whole cell lysates were immunoprecipitated (IP) with agarose-conjugated anti-HA antibody, and the precipitates were analyzed by Western blotting with anti-HA antibody. PM, membrane fractions. (E) WT and \( \text{yck1Δyck2Δ} \) cells expressing Rgt2-HA were grown in galactose (2%) to mid-log phase (\( \text{OD}_{600\text{nm}} = 1.2 - 1.5 \)) at 26 °C, and the preculture was shifted to 37 °C for various periods of time as indicated before adding glucose (2%). Then, the cells were grown for 1 h, and Rgt2-HA levels were determined by immunoprecipitation and Western blotting. (F) The cells were grown as described above (E), but the preculture was shifted to 30 °C and 37 °C before adding glucose (2%). Membrane fractions were immunoblotted with anti-HA antibody. (G,H) Western blot analysis of Rgt2-HA protein levels in WT (BY4742) and \( \text{sod1Δ} \) (KLS62) cells. Membrane fractions were immunoblotted with anti-HA (G). Whole lysates of WT, \( \text{yck1Δyck2Δ} \), and \( \text{sod1Δ} \) cells were immunoprecipitated (IP) with agarose-conjugated anti-HA antibody, and the precipitates were analyzed by Western blotting with anti-HA antibody (H). (I) The Ycs are not involved in glucose starvation-induced endocytosis and degradation of Rgt2. Western blot analysis of Rgt2-HA protein levels in WT (LRB939) and \( \text{yck1Δyck2Δ} \) (LRB1613), \( \text{end3Δ} \) (KFY127), and \( \text{yck1Δyck2Δ} \text{end3Δ} \) (KLS95) cells. Membrane fractions were immunoblotted with anti-HA. Pgk1 was used as loading control.

cyttoplasmic of the \( \text{akr1Δ} \) mutant, where, we believe, the kinases act to protect Rgt2 from degradation, suggesting that the cytoplasmic Rgt2 may be stabilized by interacting with the Ycs.
Cytoplasmic Rgt2 functions as an effective receptor for glucose signaling. Next, we examined whether the cytoplasmic Rgt2 is functional by monitoring glucose signaling markers, including Mth1 degradation and HXT expression. These markers were blocked by yck1Δyck2ts mutations, confirming that the Ycks are required for glucose signaling (Fig. 3A,C). However, Mth1-myc levels and GFP-Mth1 signals are significantly decreased in response to high glucose in akriΔ cells, indicating that the cytoplasmic Rgt2 is fully functional as a glucose receptor (Fig. 3A,B). Because extracellular glucose is unlikely to bind to the Rgt2 in the cytoplasm, we suspect that the cytoplasmic Rgt2 may directly interact with the cytoplasmic Ycks and that this interaction does not require glucose binding to Rgt2.

Of note, glucose-induced Mth1 degradation does not lead to HXT1 expression in akriΔ cells (Fig. 3C), consistent with the previous report that akriΔ cells cannot properly activate HXT1. Glucose induces HXT gene expression by ultimately effecting the release of the Rgt1 repressor from the HXT promoters. Two different
glucose-induced events occur for Rgt1 to be released from the HXT promoters via two different glucose sensing pathways: degradation of Mth1 via the GSR pathway and, phosphorylation and inactivation of Rgt1 via the Gpr1-PKA pathway. Thus, two glucose sensing pathways converge on Rgt1 to regulate expression of HXT genes.

Furthermore, PKA phosphorylation of Rgt1 does not occur until Mth1 is degraded. For this reason, we suspect that in the akr1Δ mutant Mth1 is degraded, but Rgt1 is not phosphorylated.

Rgt2 is phosphorylated in the C-terminal domain in a Yck-dependent manner. The Ycks phosphorylate and regulate the stability of many cell surface receptors and transporters. To address whether the Ycks phosphorylate Rgt2, Rgt2-HA proteins from wild-type and yck1Δyck2ts mutant cells were treated with lambda phosphatase and analyzed by Western blotting. Rgt2 from wild-type cells migrates as two bands: a major slower-migrating (upper) band and a minor faster-migrating (lower) band (Fig. 4A, lanes 1 and 3). Phosphatase treatment causes the upper band to disappear and results in the accumulation of the lower band, indicating that the band shift is due to phosphorylation (Fig. 4A, lanes 2 and 4). However, Rgt2 from yck1Δyck2ts mutant cells does not clearly show the major upper band (Fig. 4A, lane 7); instead, it exhibits faint, smeared bands that are collapsed into a single, lower band upon treatment with phosphatase (Fig. 4A, lane 8), which migrates similarly to phosphatase-treated Rgt2 from wild-type cells (Fig. 4A, lane 2).

Previous studies indicate that the C-terminal domains (CTDs) of the GSRs play an important role in glucose signaling. The CTDs of the Rgt2 and Snf3 receptors are quite dissimilar, except for a stretch of 25 amino acids (called a signaling motif) that occurs once in the Rgt2-CTD and twice in the Snf3-CTD. The Ycks catalyze phosphorylation of a serine or threonine residue in its consensus sequence (SXXS/T*, where the asterisk indicates the phosphorylated residue), and there are two clusters of potential Yck phosphorylation sites.
in Rgt2-CTD (Fig. 4B, Clusters I and II). To examine the effect of the deletion of these clusters on the stability, phosphorylation, and function of Rgt2, we expressed deletion constructs of individual motifs in wild-type and yck1Δyck2ts strains. Deletion of each cluster individually has minor or no effect on the stability of Rgt2 or its Yck-mediated phosphorylation (Fig. 4C,D). However, a deletion of up to 97-amino-acids from its C-terminus (Δ667–763) containing both clusters significantly reduces the stability of Rgt2-HA, and the resulting mutant Rgt2-HA expressed in wild-type cells migrates similarly to the protein when it is expressed in yck1Δyck2ts cells. Thus, this region may contain the Yck phosphorylation site(s).

Indeed, deletion of both clusters abolishes the ability of Rgt2 to activate the HXT1 promoter (measured by \(P_{HXT1}\)-lacZ expression), whereas deletion of either cluster alone partially perturbs Rgt2 function, resulting in ~70% (cluster I) and ~28% (cluster II) reduced HXT1 expression, respectively (Fig. 4E). Thus, Cluster I may have a more important role than Cluster II in glucose signaling. Similarly, when expressed in the \(P_{HXT1}\)-hph reporter strain, Rgt2 with the 97-amino acid deletion (Δ667–763) is unable to activate the HXT1 promoter, confirming an essential role for this region in glucose signaling (Fig. 4F).

**Yck1 interacts with the C-terminal domain (CTD) of Rgt2 in the yeast two-hybrid system.** To identify the region in Rgt2 that interacts with Yck1, we made deletions in the Rgt2-CTD (BD-Rgt2-CTD) and examined their interaction with palmitoylation–defective AD-Yck1 (lacking the palmitoylation sites 537Cys and 546Cys).
Figure 5. Rgt2 interacts with Yck1 through its C-terminal domain. (A) The yeast strain P169-4a was co-transformed with AD-YCK1 (JKP369) and either BD-RGT2 (JKP367) or indicated BD-RGT2 mutant constructs (JKP383, JKP387 and JKP416). (B) Positive interaction between AD-Yck1 and BD-Rgt2 was confirmed by expression of the GAL-HIS3 (– His + 3-AT) and GAL-lacZ reporters (β-galactosidase). (C) Western blot analysis of protein levels of wild-type Rgt2 (JKP253) and a truncated Rgt2 (Δ665–696, JKP408). Cells were grown in glucose (2%) or galactose (2%) and processed from Western blotting as described in Fig. 1A. (D) The WT strain (BY4742) strain was cotransformed the HXT1-lacZ reporter (pBM3212) with plasmid expressing either wild-type Rgt2 (JKP253) or a truncated Rgt2 (Δ665–696, JKP408). β-Galactosidase activity was assayed as described in Fig. 1B. (E,F) The P_HXT1::hph reporter strain (KLS76) expressing indicated Rgt2-HA proteins was scored for growth in a SC-2% glucose plate supplemented with 200 µg/ml hygromycin (E). The rgt2Δsnf3 double mutant (MSY441) expressing indicated Rgt2-HA proteins were spotted on 2% glucose plate supplemented with Antimycin-A (AA, 1 µg/ml) and SC-2% galactose plate (F).
The Ycks are constitutively expressed and active and are not regulated by glucose sensing receptors. (A) Mth1-9xmyc (pBM4560) tagged by immunoprecipitation was subjected to in vitro phosphorylation assays using the 7 × His-Protein A-tagged-Yck1 (7His-ProA-Yck1, pBM4536) from WT (BY4742), rgt2∆snf3∆ (YM6370), RGT2-1 (YM6545), and SNF3-1 ((YM6548) yeast strains in the presence of [γ-32P] ATP, and the radiolabeled proteins were detected by autoradiography after separating them by SDS-PAGE. The indicated yeast strains expressing 7His-ProA-Yck1 were grown in 2% glucose (+) or 2% galactose (−) as described in Fig. 1A (top). 7His-ProA-Yck1 was immunoprecipitated from cell lysates and analyzed by Western blotting using anti-Protein A antibody as a probe (bottom). (B) Yeast cells (WT and rgt2∆snf3∆) expressing 7His-ProA-Yck1 were grown in SC-2% glucose (+) medium to mid-log phase and shifted to 2% galactose (−) medium with or without cycloheximide (CHX, 50 µg/ml) for 1 h. Membrane fractions were immunoblotted with anti-HA antibody. (C) WT (BY4742) and rgt2∆snf3∆ (YM6370) strains expressing GFP-Yck1 were grown in glucose (2%) or galactose (2%) as described above and analyzed by confocal microscopy. Yeast cells were observed under the Zeiss LSM 510 META confocal laser scanning microscope. DIC and GFP fluorescence images are shown.

To examine whether YCK expression is regulated by the GSRs, we assessed protein levels of Yck1 in wild type and rgt2∆snf3∆ strains using Western blotting and confocal microscopy. Protein levels of Yck1 (7His-ProA-Yck1) are not significantly different between the wild-type and mutant strains grown with or without glucose, and treatment of the protein synthesis inhibitor cycloheximide (CHX) does not affect Yck1 expression (Fig. 6B). To further explore transcriptional regulation of the YCK1 gene, Yck1 was expressed from the MET25 promoter, which is not regulated by glucose15. We find no significant differences in the protein levels of GFP-Yck1 between wild-type and rgt2∆snf3∆ strains (Fig. 6C). These results provide evidence that the Ycks are constitutively active and that their catalytic activity is not stimulated by the GSRs.

Discussion

The Ycks are widely known as nutrient sensors that regulate cell surface abundance of many nutrient receptors and transporters33–35. The Ycks are required for glucose signaling through the GSR pathway, but their role is controversial36,37. Early studies suggest that glucose binding to the GSRs induces a conformational change in them that activates the protein kinase activity of the Ycks, which phosphorylates and inactivates the HXT corepressors Mth1 and Std1, direct targets of the GSR pathway38–42. These results indicate the role of the Yck as downstream kinases of the GSRs that transmit the glucose signal from the cell surface to the nucleus. However, here we provide evidence that the Ycks are not stimulated by the GSRs but are constitutively expressed and active. Mammalian Casein Kinases (CK1 and CK2) including CK1γ (mammalian homolog of Yck1 and Yck2) are constitutively active44, and their functions are regulated via targeting to specific subcellular locations45. Their activity could be modified by second messengers; however, this has not yet been documented in yeast. Instead, we find that Rgt2 is phosphorylated on the putative Yck consensus phosphorylation sites in its CTD in a Yck-dependent manner and that this phosphorylation increases its stability. Thus, the Ycks are likely to act upstream, but not downstream, of the GSRs.

Analysis of constitutively-signaling RGT2 mutations suggests that glucose binding to the GSRs may convert their structures from an outward-facing to an inward-facing, signaling conformation43. We believe that the glucose-bound Rgt2, which is in the signaling state, is phosphorylated and stabilized by Ycks. Interestingly, however, we also find that both Rgt2 and Yck1 expressed in an akr1A mutant are mislocalized to the cytoplasm, where Rgt2 remains stable and active as a functional receptor. Since many transporters and receptors are destroyed when removed from the plasma membrane46–51, it is plausible that the cytoplasmic Rgt2 is protected from destruction by interacting with the cytoplasmic Ycks. While this intracellular interaction may occur without...
binding of extracellular glucose to Rgt2, we cannot exclude the possibility of binding of intracellular glucose to the cytoplasmic side of the GSRs, as their glucose binding pocket may be accessible to either the outside or the inside of the cell.\(^1\)

The Ycks regulate stability of many membrane transporters in different ways; Yck activity is required for membrane trafficking of the multidrug transporter Pdr5 to the cell surface,\(^2\) whereas Yck phosphorylation of the uracil permease Fur4 facilitates its ubiquitination and internalization.\(^3\) Rgt2 is endocytosed and degraded when glucose is removed from the medium, but Yck may not be involved in this process. Rgt2 is found not to be properly localized to the plasma membrane but to be colocalized with Ycks to the cytoplasm in an akr1Δ mutant,\(^4\) suggesting a possible role for the Ycks in membrane targeting of Rgt2. One might argue that CTD phosphorylation of Rgt2 by the Ycks may be required for membrane localization of Rgt2 and that this phosphorylation does not occur in the akr1Δ mutant. However, Rgt2 from the akr1Δ mutant migrates similarly to Rgt2 from wild-type cells, indicating that the cytoplasmic Rgt2 may be fully phosphorylated in the akr1Δ mutant.\(^1\)

Methods

Yeast strains and plasmid construction. The Saccharomyces cerevisiae strains used in this study were listed in Table 1. Yeast strains were grown on YP (2% bacto-peptone, 1% yeast extract) or synthetic yeast nitrogen base medium (0.17% yeast nitrogen base and 0.5% ammonium sulfate) supplemented with appropriate amino acids and carbon sources. Genes were disrupted by homologous recombination using the Hygromycin or KanMX cassette.\(^5\)\(^6\) The plasmids used in this study were listed in Table 2. The plasmids were constructed by using standard molecular biology techniques as described previously.\(^7\) Plasmids expressing truncated forms of Rgt2-HA were constructed by QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s protocol.

Yeast membrane preparation. Membrane fractions were essentially prepared, as described previously.\(^2\)\(^5\)\(^6\) Briefly, after washing with phosphate buffer (pH 7.4), the cell pellet was resuspended in ice cold lysis buffer (100 mM Tris–Cl, pH 8, 150 mM NaCl, 5 mM EDTA) containing protease and phosphatase inhibitors and vortexed with acid-washed glass beads. After diluting the samples with the same buffer, membrane enriched fraction was collected by centrifuging the samples at 12,000 rpm for 40 min at 4 °C. The pellet was resuspended in the lysis buffer containing 5 M urea and incubated for 30 min on ice. After centrifuging at 14,000 rpm for 40 min at 4 °C, the pellet was dissolved in SDS buffer (50 mM Tris–HCl (pH, 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol).

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were carried out as described previously.\(^8\) Briefly, yeast cells were disrupted by vortexing with acid-washed glass beads in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.5, 140 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate) containing protease and phosphatase inhibitors (10 mM Na-pyrophosphate, 200 µM Na-orthovanadate,

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**Table 1. Yeast strains used in this study.**

| Strain     | Genotype                                      | Source |
|------------|-----------------------------------------------|--------|
| BY4742     | MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ              |        |
| YM6370     | BY4742 rgt2-kanMX snf3::kanMX                 |        |
| LRR939     | MATa his3 leu2 ura3Δ2                         |        |
| LRR1613    | LRR939 yck1::KanMX yck2-2ts                   |        |
| KFY127     | MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 end3::KanMX |        |
| KLS95      | LRR1613 end3::KanMX                          | This study |
| MSY401     | MATa ura3Δ2 leu2Δ1 his3Δ2Δ0 trpl1::Δm3      |        |
| MSY441     | MATa ura3Δ2 leu2Δ1 his3Δ2Δ0 trpl1::Δm3 snf3::hisG rgt2::HIS3 |        |
| KLS76      | MATa ura3Δ2 leu2Δ1 his3Δ2Δ0 trpl1::Δm3 snf3::hisG rgt2::HIS3 P<sub>ext</sub>·hph |        |
| KLS61      | MATa his3Δ1 leu2Δ0 ura3Δ0 met15A akr1::KanMX | This study |
| KLS62      | MATa his3Δ1 leu2Δ0 ura3Δ0 met15A sod1::KanMX  | This study |
| YM6545     | MATa his3Δ1 leu2Δ0 ura3Δ0 met15A RGT2-1       |        |
| YM6548     | MATa his3Δ1 leu2Δ0 ura3Δ0 met15A SNF3-1       |        |
| P69-4a     | MATa trpl901 leu2-3.112 ura3-52 his3-200 gal4gal80A LYS2·GAL1·HIS3 GAL2·ADE2 met2·GAL7·lacZ |        |

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50 mM Na-flouride). The resulting cell lysates were incubated with appropriate antibodies at 4 °C for 3 h and further incubated with protein A/G-conjugated agarose beads at 4 °C for 1 h. The agarose beads were washed three times with RIPA buffer and boiled in SDS–PAGE buffer. The eluted proteins were subjected to Western blot analysis. For Western blotting, proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were incubated with appropriate antibodies in TBST buffer (10 mM Tris–HCl, pH, 7.5, 150 mM NaCl, 1% Tween-20), and proteins were detected by the enhanced chemiluminescence (ECL) system (BioRad, Hercules, CA, USA) (Supplementary Information).

\section*{In vitro protein kinase assay.} In vitro protein kinase assay was performed as described previously\cite{21}. Mth1-9xMyc and 7X His-Protein A-tagged Yck1 were affinity-purified using agarose beads as described previously\cite{19} and mixed in 50 μl of kinase buffer containing 0.5 μCi of [γ 32P] ATP, 100 μM ATP, 10 mM MgCl2 for 30 min. After washing the beads with the kinase buffer containing 0.5 M NaCl, the proteins were eluted by boiling the beads in SDS-sample buffer for 5 min. The eluted proteins were resolved by SDS-PAGE and detected by autoradiography. Each set of in vitro kinase assays was independently repeated twice.

\section*{Yeast two-hybrid assay.} To construct Gal4 DNA-binding domain hybrids (GAL4-DBD-RGT2), the C-terminal domain of RGT2 (encoding amino acids 546–763) was amplified by PCR using JKP253 as a template, and the PCR products were incorporated into the GAL4-DBD plasmid\cite{56}. These plasmids were combined with the GAL4 activation domain hybrid (GAL4-AD-YCK1) and used to transform the yeast strain PJ69-4A\cite{56} to Leu+Trp+. Cells were grown on selective medium (SC-leu-trp) medium lacking histidine (SC-leu-trp-his + 20 mM 3-AT) to detect expression of the \textit{GAL-HIS3} reporter or medium containing X-gal (SC-leu-trp + X-gal) to assay GAL-lacZ report gene expression.

\section*{β-Galactosidase assay} β-Galactosidase activity assays were performed using the yeast β-galactosidase assay kit (Pierce) according to the manufacturer’s instructions\cite{10}. Results were presented in Miller Units ((1000 × A420)/(T × V × A600)), where A420 is the optical density at 420 nm, T is the incubation time in minutes, and V is the volume of cells in milliliters. The reported lacZ activities are averages of results from triplicate of usually three different transformants.

\section*{Confocal microscopy.} GFP-fusion proteins expressed in yeast cells were visualized using a Zeiss LSM 510 META confocal laser scanning microscope with a 63× Plan–Apochromat 1.4 NA Oil DIC objective lens (Zeiss)\cite{32}. All images documenting GFP localization were acquired with the Zeiss LSM 510 software version 3.2.

\begin{table}
\centering
\begin{tabular}{|l|l|l|}
\hline
Plasmid & Description & Source \\
\hline
JKP293 & pUG34-P\textsubscript{AUG25}-GFP-Rgt2 & \cite{25} \\
KFP69 & pPAD80, C-terminal 3xHA fusion & \cite{24} \\
JKP253 & pPAD80-P\textsubscript{ACT7}-Rgt2-3xHA & \cite{25} \\
JKP408 & JKP253 Δ665–696 & This study \\
JKP461 & JKP253 Δ684–690 & This study \\
JKP462 & JKP253 Δ667–673 & This study \\
JKP468 & JKP253 Δ721–763 & This study \\
JKP447 & JKP253-prRS316 & This study \\
JKP450 & JKP408-prRS316 & This study \\
JKP465 & JKP461-prRS316 & This study \\
JKP467 & JKP462-prRS316 & This study \\
JKP469 & JKP468-prRS316 & This study \\
JKP369 & pGAD-Yck1(-CC) & This study \\
JKP367 & pGBD-Rgt2-CTD (546–763) & This study \\
JKP383 & pGBD-Rgt2 (545–624) & This study \\
JKP387 & pGBD-Rgt2 (625–763) & This study \\
JKP416 & JKP367 Δ665–696 & This study \\
JKP438 & pUG34-P\textsubscript{AUG25}, GFP-Yck1 & \cite{31} \\
\hline
\end{tabular}
\caption{Plasmids used in this study.}
\end{table}

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Author contributions
J.K. designed and performed the research, analyzed data, and wrote the paper. D.B., R.R., E.M., L.M., and D.J. performed the research. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information

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