The Yeast Glucose Sensing Receptor is Stabilized by Interaction with Casein Kinases

Emma E. Mohler

A Senior Thesis submitted in partial fulfillment
of the requirements for graduation
in the Honors Program
Liberty University
Spring 2022

Acceptance of Senior Honors Thesis

This Senior Honors Thesis is accepted in partial fulfillment of the requirements for graduation from the Honors Program of Liberty University.

____
Jeong-Ho Kim, Ph.D.
Thesis Chair

____
Gregory Raner, Ph.D.
Committee Member

____
Cindy Goodrich, Ed.D.
Assistant Honors Director

____
Date

Abstract

Many metabolic diseases are rooted in the inability to process glucose or regulate its uptake. These processes can be explored using yeast as a model. Rgt2 is a glucose sensing receptor in yeast, and it detects glucose concentration outside the cell. This receptor is present on the plasma membrane in high glucose conditions but absent in low glucose. Western blot and a yeast two-hybrid assay were used to investigate the relationship between Rgt2 and the plasma membrane-tethered yeast casein kinases, Yck1 and Yck2. This research demonstrated that in high glucose, Rgt2 is stabilized on the plasma membrane by interaction with Yck1 and Yck2. In response to glucose, the Ycks likely phosphorylate the Rgt2 CTD between amino acids 665-696.

The Yeast Glucose Sensing Receptor is Stabilized by Interaction with Casein Kinases

Glucose as an Energy Source

Glucose is a key nutrient in the metabolic pathways of organisms as diverse as bacteria and human beings. It can be converted into energy quickly or used biosynthetically (Towle, 2005). It is a six-carbon carbohydrate that can isomerize between a ring structure and a linear structure. Glucose is not the only six-carbon carbohydrate; its close cousins include other hexoses, such as galactose, fructose, and mannose (Hantzidiamantis & Lappin, 2021). It is often found in polymerized groups of sugars, either in combination with itself or with other carbohydrates to make substances such as common table sugar and starch. Along with other six carbon sugars, it is a product of photosynthesis in plants (Rolland, Winderickx, & Thevelein, 2001).

The metabolism of glucose is crucial to human health. Disorders such as diabetes and cancer center around glucose intake. In diabetes, the cells are unable to properly take glucose into the cells due to deficiencies in insulin production or response. Cancer cells are known to consume glucose at an unusually quick rate, and they do so through an inefficient process, relying on glycolysis instead of fully processing the components of glucose through cellular respiration; this phenomenon is called the Warburg effect (Kim, Jeong-Ho, Roy, Jouandot, & Cho, 2013).

In order to better understand the processes behind glucose metabolism in humans, yeast are often studied as a model organism. In yeast, glucose is preferred for energy production because of its convenient usage in glycolysis for the generation of ATP. Yeast also mirror cancer cells in that they prefer to only use glucose though the glycolysis pathway. This inefficiency

leads to a high glucose intake requirement and justifies the complex system of glucose transportation and regulation in yeast (Kim, Jeong-Ho et al., 2013).

Yeast Hexose Transporters and Their Regulation

Due to the importance of glucose for energy, yeast have multiple hexose transporters of varying affinity for glucose. The system for their regulation and expression is complex and adaptable (Busti, Coccetti, Alberghina, & Vanoni, 2010). In the yeast genome, there are 20 genes that are either known to be glucose transporters or are genetically similar (Özcan, Sabire & Johnston, 1999). Within those 20 genes, there are six known glucose transporters, one galactose transporter, and two glucose sensing receptors. (Özcan, Sabire & Johnston, 1999)

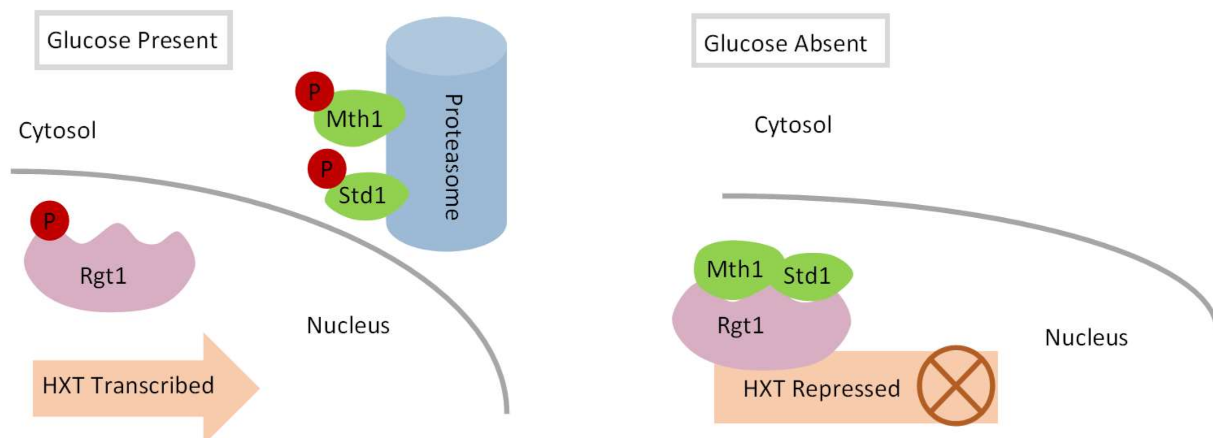
Yeast cells control expression of the glucose transporters according to the concentration of glucose available (Özcan, Sabire & Johnston, 1999). For example, the transporter HXT1 is abundant in high glucose conditions, over 1%, while HXT2 is produced for glucose conditions as low as 0.1%, because it is highly sensitive to glucose (Ozcan & Johnston, 1995). The variety of transporters allow the cell to adapt to many glucose conditions and efficiently take in the glucose from the environment.

There are two main systems of repressors for glucose transporters: the Rgt1 repressor with its corepressors, and the Mig1 repressor pathway (Özcan, Sabire & Johnston, 1999). The glucose transporters HXT1-4 are repressed by Rgt1, which binds to the HXT promoters and is localized in the nucleus. When phosphorylated, Rgt1 is unable to bind to the promoters, and the HXT gene is transcribed (Flick et al., 2003; Kim, J. -H, Polish, & Johnston, 2003). Rgt1 binds to Mth1 and Std1, which are corepressors with Rgt1 and are necessary for it to bind to the promoter and inhibit HXT transcription (Figure 1) (Lakshmanan, Mosley, & Özcan, 2003).

Mth1 and Std1 are degraded when in their phosphorylated form by ubiquitination (Kim, Jeong-Ho, Brachet, Moriya, & Johnston, 2006). Mth1 is degraded when glucose is present, deactivating Rgt1 and allowing HXT production. Although it is also phosphorylated and degraded when glucose is present, the transcription of Std1 is activated by glucose, allowing for the pathway to be quickly turned off when the available glucose is used up (Kim, Jeong-Ho et al., 2006). Once Mth1 and Std1 are ubiquitinated and degraded in the 26S proteasome, Rgt1 is phosphorylated by the cAMP-dependent protein kinase A, allowing the HXT genes to be transcribed (Figure 1) (Kim, J. -H & Johnston, 2006).

Figure 1

HXT Repression by Rgt1 and its Corepressors



Note. When glucose is present, Rgt1 is phosphorylated and deactivated, and Mth1 and Std1 are phosphorylated and degraded in the proteasome. As a result, the HXT genes are transcribed. When glucose is absent, Mth1 and Std1 coordinate with Rgt1 in the nucleus to repress HXT transcription. Created by Emma Mohler.

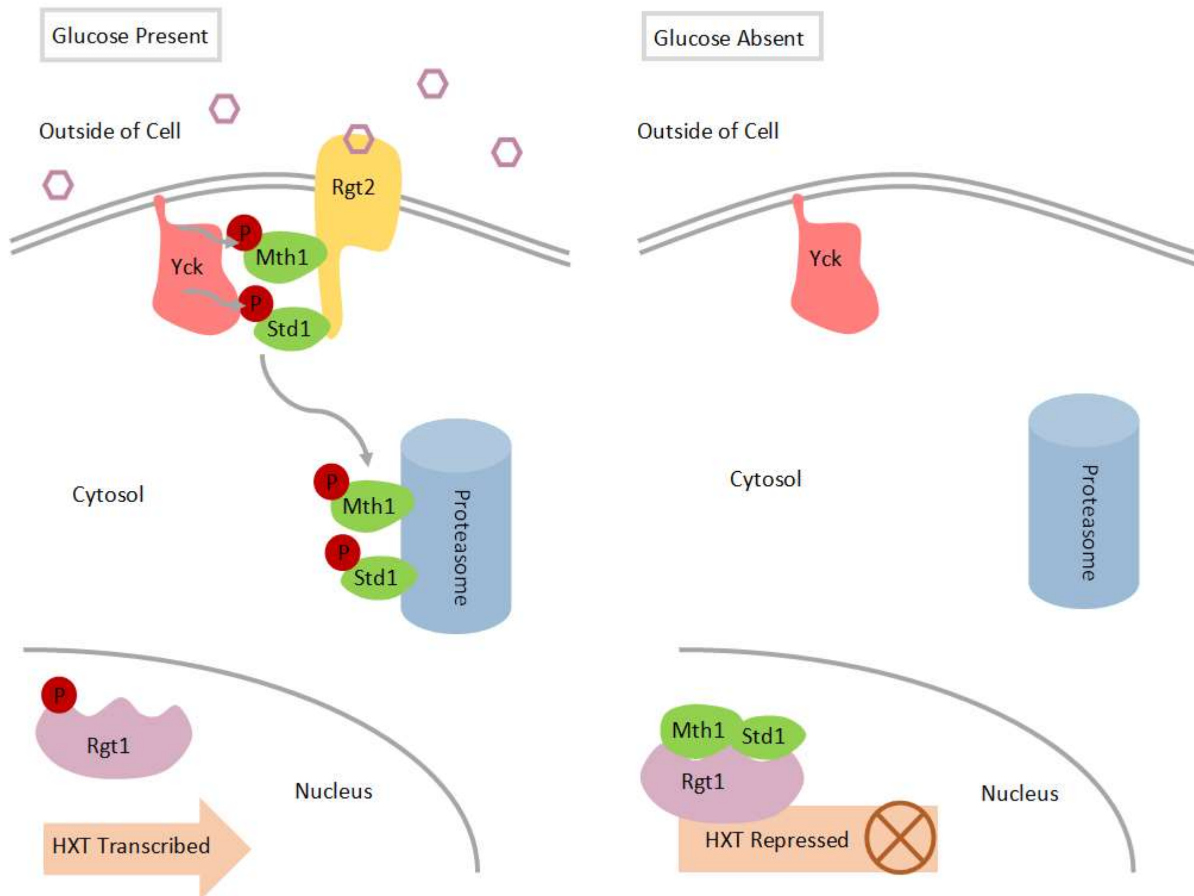
Glucose Sensing Receptors Rgt2 and Snf3

There are two glucose sensing receptors: Rgt2 and Snf3. These are paralogs of the hexose transporters and also bind to glucose (Towle, 2005). Glucose sensing receptors bind extracellular glucose and send an intracellular signal leading to HXT expression for the import of glucose (Scharff-Poulsen, Moriya, & Johnston, 2018). Rgt2 activates glucose transporters in high glucose conditions, while Snf3 works in low glucose conditions (Kim, Jeong-Ho & Rodriguez, 2021). The two glucose sensing receptors Rgt2 and Snf3 trigger expression of transporters with affinities appropriate to the concentration of glucose. When glucose is high, Rgt2 causes expression of low affinity transporters, such as HXT1, which are effective in high concentrations of glucose. Snf3 signals for expression of high affinity transporters, which are needed when glucose concentrations are low (Kim, Jeong-Ho et al., 2013).

The glucose sensing receptors send the intracellular signal by interaction with Mth1 and Std1 (Schmidt et al., 1999). The C terminal domains of both Rgt2 and Snf3 have been demonstrated to interact with Mth1 in a glucose dependent manner (Lafuente, Gancedo, Jauniaux, & Gancedo, 2000). The interaction depends on the conformation of Rgt2 and Snf3. When they are glucose bound, the glucose sensing receptors undergo a conformational change that allows interaction with Mth1 and Std1 (Moriya & Johnston, 2004). The glucose sensing receptors have also been found to interact with the yeast casein kinases. Rgt2 and Snf3 bring Mth1 and Std1 in close proximity to the yeast casein kinases, which phosphorylate Mth1 and Std1. They are then targeted for degradation by the 26S proteasome, allowing for deactivation of Rgt1 and corresponding expression of the HXT genes (Figure 2) (Moriya & Johnston, 2004).

Figure 2

Signal Transduction by Rgt2 to Control HXT Expression



Note. When glucose is present, Rgt2 recruits Mth1 and Std1 for phosphorylation by Yck. This targets them to the proteasome, triggering HXT transcription. When glucose is absent, Rgt2 is endocytosed and not available for this process. Mth1 and Std1 coordinate with Rgt1 to repress HXT expression. Created by Emma Mohler.

It has been demonstrated that Rgt2 triggers expression of glucose transporters appropriate for high glucose concentrations, and that Snf3 leads to transporters for low glucose condition (Kim, Jeong-Ho et al., 2013). This could be explained by Rgt2 and Snf3 having different affinities for glucose (Özcan, S., Dover, Rosenwald, Wölfl, & Johnston, 1996). However, the

glucose affinities of Rgt2 and Snf3 have not been confirmed, and it is not known for certain whether the glucose sensing receptors bind glucose directly, although this is a reasonable assumption (Kim, Jeong-Ho & Rodriguez, 2021).

A second model for the different signaling capabilities of Rgt2 and Snf3 has been suggested. Rgt2 is transcribed constitutively across glucose concentrations. Snf3, however, is controlled by transcription, only transcribed in low glucose conditions, as well as at a very low level in the absence of glucose (Kim, Jeong-Ho & Rodriguez, 2021). The low level of Snf3 remaining in glucose starved conditions helps to restore HXT expression when glucose becomes accessible again. When Snf3 is expressed from a constitutive promoter instead of its native promoter, it follows the expression pattern of Rgt2 (Kim, Jeong-Ho & Rodriguez, 2021). Additionally, it results in the expression of HXT1, which is the transporter that Rgt2 induces. Snf3 and Rgt2 exhibit interchangeable behavior when both are expressed constitutively (Kim, Jeong-Ho & Rodriguez, 2021).

Instead of transcriptional regulation, Rgt2 is controlled by posttranslational regulation, being removed from the plasma membrane by the end3 endocytosis pathway in the absence of glucose (Figure 2) (Kim, Jeong-Ho & Rodriguez, 2021). When the end3 gene is deleted, Rgt2 remains on the cell surface regardless of glucose concentration. Interestingly, Snf3 cell surface abundance is not affected by the deletion of the end3 gene, indicating that it is not controlled by this pathway. The different roles of Rgt2 and Snf3, then, can be attributed to differences in protein expression and regulation (Kim, Jeong-Ho & Rodriguez, 2021).

Galactose is a sugar that differs from glucose by the position of a hydroxyl group at only one carbon: C4. Surprisingly, Rgt2 is specifically sensitive to only glucose (Kim, Jeong-Ho & Rodriguez, 2021). When glucose-containing media is replaced with media with only galactose,

Rgt2 is quickly degraded. On the other hand, when glucose is added back, levels of Rgt2 are quickly restored (Kim, Jeong-Ho & Rodriguez, 2021). Additionally, Rgt2 is not stabilized by the presence of other carbon sources like ethanol and glycerol, even though the level of mRNA remains constant. This suggests that Rgt2 is stabilized only when bound to glucose and confirms that its abundance on the plasma membrane is controlled by endocytosis and not by transcriptional regulation (Kim, Jeong-Ho & Rodriguez, 2021).

The pattern of endocytosis of Rgt2 is unusual compared to other nutrient sensors in yeast. Typically, nutrient sensors are highly present when the nutrient is absent, so that when the nutrient becomes available, a conformation change signals for downstream transporters or other necessary proteins. Once the nutrient has been absorbed as much as needed by the cell, the sensors are degraded to prevent excess intake of the nutrient (Kim, Jeong-Ho & Rodriguez, 2021). Surprisingly, Rgt2 appears to work opposite of this general pattern. When glucose levels are high, Rgt2 remains consistently stable on the plasma membrane, whereas it is degraded in the absence of glucose. Rgt2 seems to be a representation of the extracellular glucose concentration. This pattern also carries over to glucose transporters signaled for by Rgt2: HXT1 and HXT3 (Kim, Jeong-Ho & Rodriguez, 2021).

Yeast Casein Kinases and Phosphorylation

The yeast casein kinases 1 and 2 (Ycks) are two kinases involved in glucose signaling. They are plasma membrane associated kinases, attached by a palmitoyl group on the C terminus. They are known to be involved in phosphorylating multiple different nutrient permeases (Snowdon & Johnston, 2016). The palmitoyl transferase responsible for attaching the Ycks is called Akr1 (Kim, Jeong-Ho et al., 2013). The Ycks are essential for cell survival, so a knockout strain in nonviable. The Akr1 knockout, then, can be used to simulated loss of the Ycks, because

Yck is not properly localized to the plasma membrane and therefore loses its ability to interact as usual with plasma membrane bound proteins (Pasula, Chakraborty, Choi, & Kim, 2010).

The yeast casein kinases have been shown to interact with Rgt2 and Snf3 (Moriya & Johnston, 2004). For the purpose of glucose signaling, they function equivalently. Because the glucose sensing receptors interact with Mth1 and Std1, they are in a prime location to be phosphorylated by the Ycks, and this has been shown to be the mechanism of phosphorylation of Mth1 and Std1 (Snowdon & Johnston, 2016). Due to this role in the signaling process, the Ycks are essential for HXT expression (Snowdon & Johnston, 2016).

There have been multiple suggestions for the role of the Ycks in glucose signaling. One model was that glucose-bound Rgt2 and Snf3 activate the Ycks. Once activated, the Ycks phosphorylate Mth1 and Std1, leading to HXT expression (Snowdon & Johnston, 2016). Recent work, however, has suggested that Yck works upstream or at the same level as Rgt2 and Snf3. Yck does not appear to be activated by the glucose sensing receptors but may work alongside them or on them (Snowdon & Johnston, 2016).

The possibility of Yck phosphorylation of Rgt2 and Snf3 fits within this model. The Ycks phosphorylate their targets at a consensus sequence of SXXS/T, phosphorylating the last serine or threonine in the sequence. This phosphorylation target sequence is present in both Rgt2 and Snf3, as well as Mth1 and Std1. In Rgt2, it is present in two clusters (Kim, Jeong-Ho et al., 2022). The role of the Ycks in interacting with the glucose sensing receptors Rgt2 and Snf3 has not been satisfactorily determined to this point.

This study sought to investigate the relationship between Rgt2 and the Ycks. The dependence of Rgt2 on the Ycks for expression on the plasma membrane was examined using Western blot with knockout strains simulating Yck deactivation or delocalization. Additionally,

the interaction of Yck with one of the two clusters of the Yck consensus sequence present on the Rgt2 C-terminal domain (CTD) was studied with a yeast two-hybrid assay. The work examined here was the contribution of the author to the publication, "Casein kinases are required for the stability of the glucose-sensing receptor Rgt2 in yeast" by Jeong-Ho Kim et al. in Scientific Reports, January 31, 2022.

Methods

Yeast Strains and Plasmids

The yeast strains and plasmids used in this research are listed in the following Tables 1 and 2, respectively. These strains and plasmids were synthesized by or obtained by Dr. Jeong-Ho Kim of Liberty University (Kim, Jeong-Ho et al., 2022).

Table 1

Yeast Strains Used

Name	Strain	Genotype	Description
WT	BY4742	MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ	Wild Type Yeast
Yck WT	LRB939	MATα his3 leu2 ura3-52	Wild Type with Background of Yck TS
Yck TS	LRB1613	LRB939 yck1::KanMX yck2-2ts	Yck Temperature Sensitive
End3Δ	KFY127	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 end3::KanMX	End3 Knockout
Yck TS end3Δ	KLS95	LRB1613 end3::KanMX	Yck TS with end3 Knockout
Akr1Δ	KLS61	MATα his3Δ1 leu2Δ0 ura3Δ0 met15Δ akr1::KanMX	Akr1 Knockout
Y2H	PJ69-4a	MATα trpl-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-1acZ	Yeast Two-Hybrid Assay Strain

Table 2

Plasmids Used

Name	Description	Gene of Interest
JKP253	pPAD80-P_{RGT2}-Rgt2-3xHA	Rgt2 tagged with HA
JKP293	pUG34-P_{MET25}-GFP-Rgt2	Rgt2 tagged with GFP
JKP369	pGAD-Yck1(-CC)	Yck1 on activation domain for Y2H
JKP367	pGBD-Rgt2-CTD (546-763)	Rgt2 CTD on binding domain for Y2H
JKP416	JKP367 Δ665-696	Rgt2 CTD without PEST sequence for Y2H

Yeast Transformation with Plasmids

The yeast strain of interest was inoculated overnight in YPD liquid media (2% bacto-peptone, 2% glucose, 1% yeast extract). Cells were washed twice in water and once in 100 mM lithium acetate (LiAc). They were incubated in 100mM LiAc for 15-30 minutes. After centrifuging at 14,500 rpm for 30 seconds to pellet the cells, the following transformation mix

was added: 240 µl of PEG (50% w/v), 36 µl of 1.0M LiAc, 50 µl of single-stranded carrier DNA (2.0 mg/ml), and 31 µl of sterile deionized water.

Cells were resuspended, and 3 µl of plasmid DNA (0/1-10 µg) was added and gently mixed in by inversion or brief vortexing. This was incubated for 20-30 minutes at room temperature, and heat shocked for 20-25 minutes at 42°C. After pelleting with 6,000 rpm for 30 seconds, the transformation mix was removed and 200 µl sterile water added. This was plated on the appropriate drop out media with 2% glucose. Colonies of successful transformants were noted after 2-3 days and amplified on fresh drop out plates, to be used for Western blot or the yeast two-hybrid assay.

Plasma Membrane Fraction Sample Preparation

Yeast samples were grown in SC-Leu or SC-His drop out media with glucose (1% glucose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and selective amino acid supplements) to optical density (OD) 1.0-1.3. 10 ml samples were centrifuged at 3,500 rpm for 5 minutes. The cells were resuspended in drop out media plus a carbon source of 2% for 45 minutes to 1 hour. After centrifuging the sample and discarding the media, the cells were frozen at -60°C.

The cells were thawed and resuspended on ice in 400 ul lysis buffer with protease inhibitors (1 M ammonium acetate, 150 mM NaCl, 30 mM Tris-HCl pH 7.5, 10 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and the protease inhibitor cocktail complete (11836145001; Roche, Basel Switzerland)) with 300 ul glass beads (G8772 Sigma). The tube was intensely vortexed for 3 minutes and 45 seconds, with four 1-minute breaks on ice. The mixture was centrifuged at 3,000 rpm for 3 minutes, and 200 ul supernatant was transferred to a new tube.

600 ul lysis buffer with 5 M urea was added and incubated on ice for 15min. The new mixture was centrifuged for 45 minutes at 14,500 rpm at 4°C. The supernatant was discarded and the pellet resuspended in 200 ul lysis buffer with 10% trichloroacetic acid (TCA) for 10 minutes. After centrifugation, the pellet was resuspended in 50-60 ul Lammelli buffer with beta-mercaptoethanol. The sample was centrifuged once more, and the supernatant was used for Western blot.

Western Blot

Western blot is a protein detection method that utilizes antibodies to visualize proteins that are adhered to a membrane. Protein samples are prepared in buffer containing sodium dodecyl sulfate (SDS), which denatures the proteins and gives a net negative charge proportional to protein size. The protein is separated based on size using polyacrylamide gel electrophoresis (SDS-PAGE), which draws the proteins through a gel matrix at a speed corresponding to protein size.

The protein is transferred to a protein-binding membrane, maintaining the size-relative positions achieved on SDS-PAGE. Primary antibody is applied that corresponds to the protein of interest; secondary antibody is added that binds to the primary antibody and provides a detection method, such as fluorescence or chemical reactivity. The membrane is then imaged, giving a qualitative and comparative measurement of the amount of the protein of interest in the sample.

Because Western blot is qualitative, one method for standardization is having a loading control. This is another protein that is known to be constitutively expressed across a wide variety of growth conditions. It is detected along with the protein of interest to show that sample concentrations are comparable between samples and confirms the validity of the results. For

some of the figures in this study, the loading control used was the protein Pgk, a constitutively expressed structural protein.

Another method for ensuring equal protein concentration across samples is directly measuring protein concentration through the Bradford assay. The Bradford reagent is a protein-binding dye that turns blue when bound to protein but is otherwise a dull brown or orange. The intensity of color is linearly related to protein concentration within the linear range, which can be found using a protein standard and generating a calibration curve. This method was also used for some of the data in this study.

The following protocol was used for SDS-PAGE to Western blot. 10-20 ul samples were loaded into a 7.5% or 10% polyacrylamide gel and run for 1 hour to 1 hour and 30 minutes at 100 V according to the standard BioRad SDS-PAGE protocol, until the blue solvent line reached the bottom of the gel. The gel was briefly rinsed in running buffer, and the PVDF membrane was soaked in methanol for 2 minutes to activate. The protein was transferred to the membrane using the BioRad TurboTransfer system.

The membrane was blocked in TBST with 5% milk (0.137 M NaCl, 20 mM Tris base, 5% w/v nonfat dry milk, 1% v/v Tween 20) for 30 minutes. Primary antibody solution was prepared by adding 10 ul primary antibody (anti-HA, anti-GFP, or anti-Pgk) to 10 ml TBST with 5% milk. The membrane was incubated in primary antibody solution for 1 hour. It was washed in TBST for 5 minutes three times, and secondary antibody solution (2 ul antibody in 10 ml TBST with milk) was added for 1 hour. After washing with TBST for 5 minutes three times, the membrane was imaged. Imaging was done with ECL substrates and a BioRad ChemiDoc imager. ECL substrates were added to the membrane in a 1:1 ratio of the two solutions for 1 minute in the dark before imaging.

The Yeast Two-Hybrid Assay between Rgt2 and Yck

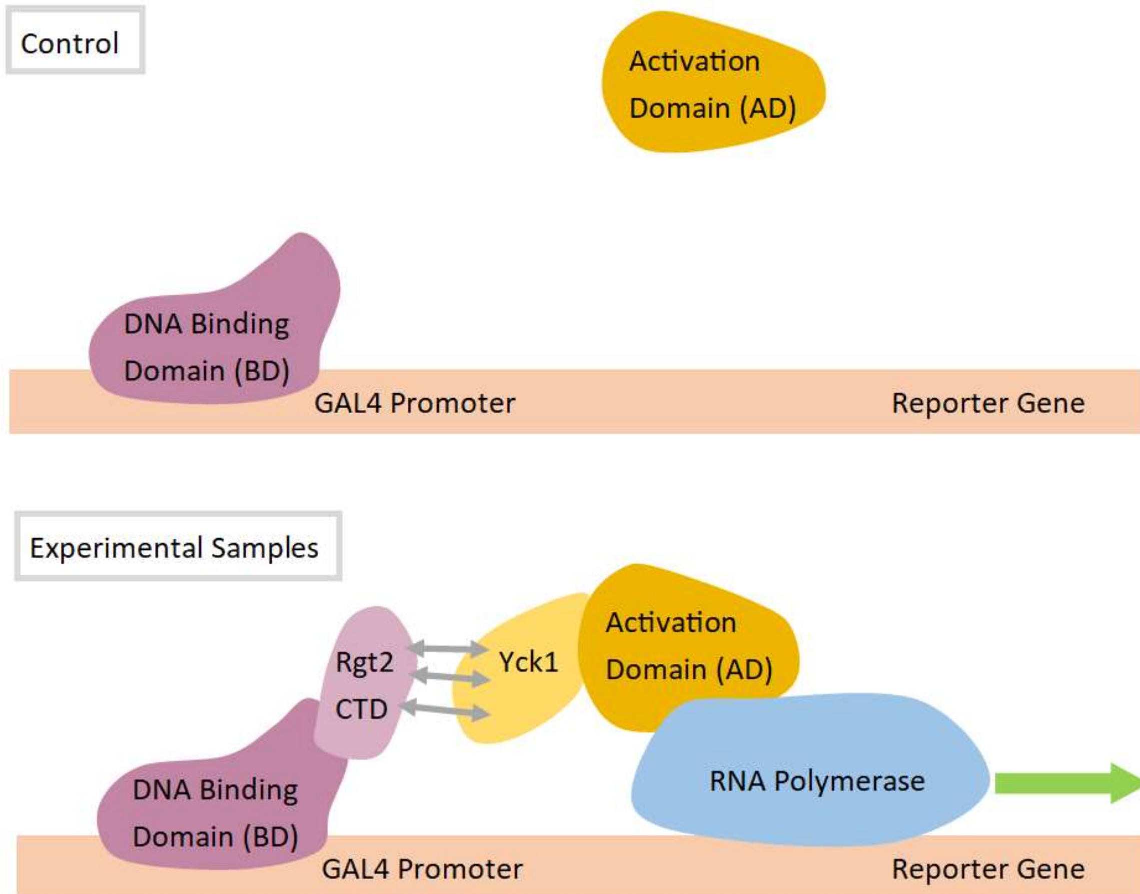
A yeast two hybrid assay was used to measure Rgt2 and Yck interaction. In this assay, the promoter of the GAL4 gene and its activating protein are used to measure the interaction between two proteins of interest. The GAL4 activator is split into its two parts: the DNA binding domain (BD), and the activation domain (AD). The two proteins of interest are each fused to one subunit of the activator protein (Figure 3).

If the two proteins interact, the activator is able to express the reporter gene, giving the cell various abilities that can be detected using specialized media (Figure 3). The strain to be used was developed with the GAL4 promoter inserted before several reporter genes: beta-galactosidase, -Ade, and -His. When activated, -Ade and -His allow cell survival in nutrient deficient media or media containing enzyme inhibitors, and beta-galactosidase produces a blue pigment in the presence of X-gal.

The yeast two hybrid assay was used for three sample constructs. The Y2H strain was transformed with two different plasmids each time. One sample simply contained the original BD (binding domain) and AD (activation domain) cleaved, with no proteins of interest added, as a negative control. Another sample had the Rgt2 C terminal domain (CTD) fused to the BD and Yck1 fused to the AD (Figure 3). A third sample fused the Rgt2 CTD to the BD but with amino acids 665-696 removed, as well as Yck1 with the AD.

Figure 3

Yeast Two-Hybrid Assay Illustration



Note. The C-terminal domain of Rgt2 was fused to the DNA binding domain (BD), and Yck1 was fused to the activation domain (AD). The diagram illustrates what would occur if Rgt2 and Yck interact. The control situation is also illustrated. Created by Emma Mohler.

Once these transformations were done, the cells were plated on specialized media. SC-Ade-Leu-Trp was a drop out media without adenosine, a nucleotide needed for cell reproduction. In the Y2H strain, the adenosine production gene is controlled by the GAL4 promoter, linking cell survival with the ability to turn on the adenosine gene through the interaction of the proteins

of interest. SC-Leu-Trp-His+50mM 3AT contained 3-amino-1,2,4-triazole, a competitive inhibitor of histidine, an essential amino acid for the Y2H strain. The histidine gene is controlled by the same GAL4 promoter and must be activated enough to out-compete the 3AT absorbed by the cell. SC-Leu-Trp+X-gal detected beta-galactosidase activity, with generates a blue color. These samples were also tested in a quantitative beta-galactosidase assay by Pierce, according to manufacturer's instructions.

Results

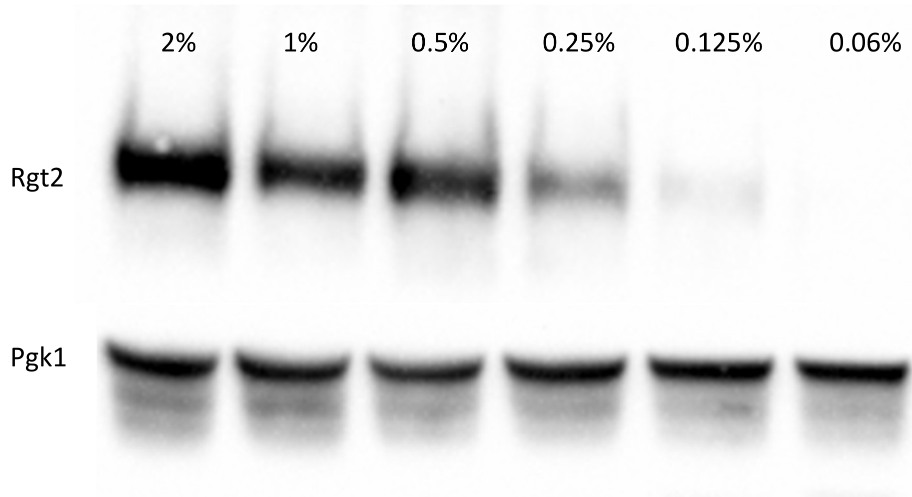
The following results have also contributed to the paper by Jeong-Ho Kim et al, "Casein kinases are required for the stability of the glucose-sensing receptor Rgt2 in yeast," published by Scientific Reports on January 31, 2022.

Confirmation of the Wild Type Expression of Rgt2

As previously reported (Kim, Jeong-Ho & Rodriguez, 2021), the presence of Rgt2 on the plasma membrane was observed to correlate clearly with glucose concentration. When glucose concentration was above 0.5%, Rgt2 was detected strongly in the plasma membrane. Concentrations of lower than 0.125% yielded poor detection of Rgt2 (Figure 4).

Figure 4

Wild Type Expression of Rgt2 Across Glucose Conditions



Note. WT+253 yeast were grown in SC-Leu+1% glucose to OD 1.36. Samples were incubated in SC-Leu+glucose for 45 minutes, the percentages of glucose being 2%, 1%, 0.5%, 0.25%, 0.125%, and 0.06%. 15 ul of each sample was loaded into a 7.5% gel and run at 100 V. The primary antibody used for Western blot was HA antibody, and secondary was HRP antibody. Cellular fractions of the samples were also run on Western blot with Pgk primary antibody, as a loading control. Rgt2 decreases in prevalence in the plasma membrane fraction as the glucose concentration decreases. Created by Emma Mohler.

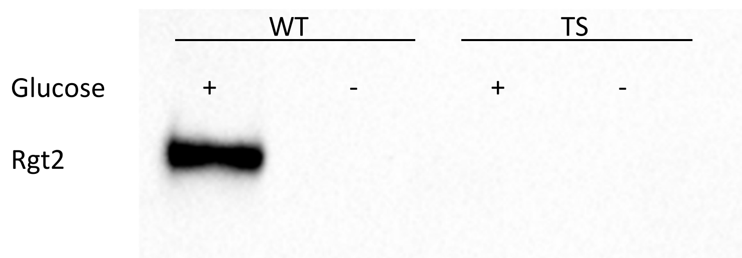
Rgt2 Not Stable in the Plasma Membrane when Yck is Deactivated

Western blotting was used to detect Rgt2 levels in various strains that disrupt Yck and Rgt2 interaction. This was done in a Yck temperature sensitive strain. Yck is inactivated when the yeast are grown at 37°C, shown in figures 5 and 6, but is still active when grown at room temperature. This allows the yeast to grow and survive, but the Ycks can be deactivated for a time to study their effects. When Yck was inactivated in the heat sensitive strain and compared

to the wild type expression of Rgt2, Rgt2 was not present on the plasma membrane, even when glucose was readily available to the cell. Figures 5 and 6 show that this observation persisted when Rgt2 was tagged with two methods on its C or N terminus, with hemagglutinin (HA) and green fluorescent protein (GFP).

Figure 5

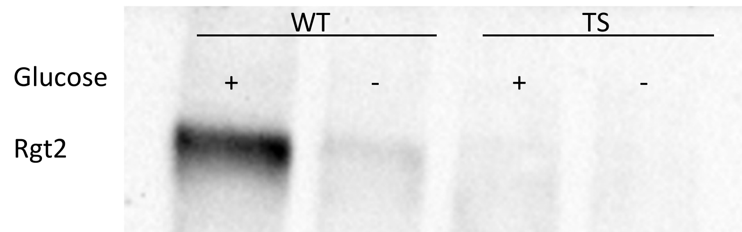
Rgt2 Expression in Yck TS with HA Tagging



Note. Yck WT +253 (WT) and Yck TS +253 (TS) yeast were grown overnight to OD 1.3. The TS sample was heat shocked at 37°C for 30 minutes. Samples were incubated in 2% glucose (+) or galactose (-) for 1 hour. The plasma membrane fractions were prepared per protocol. Protein concentration was normalized using the Bradford assay. 15 ul of each sample was loaded into a 10% gel and run at 100 V. The primary antibody used for Western blot was HA antibody, and secondary was HRP antibody. While active in the wild type sample, Rgt2 is not observed in the plasma membrane fraction when Yck is inactivated by heat. Created by Emma Mohler.

Figure 6

Rgt2 Expression in Yck TS with GFP Tagging

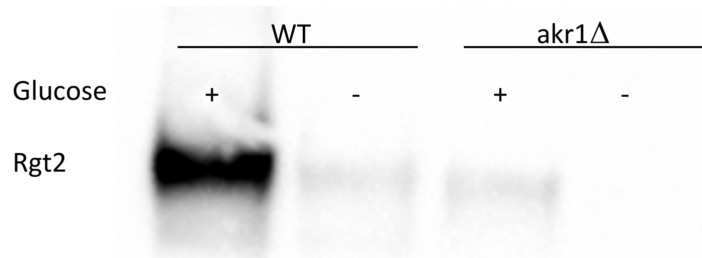


Note. Yck WT+293 and Yck TS+293 yeast were grown in SC-Leu+1% glucose to OD 1.0. The TS strain was then heat shocked at 37°C for 30 minutes. The samples were put in 2% glucose (+) or galactose (-) for 45 minutes. The plasma membrane fractions were prepared per protocol. 15 ul of each sample was loaded into a 10% gel and run at 100 V. The primary antibody used for Western blot was GFP antibody, and secondary was HRP antibody. Rgt2 with GFP tagging is also not seen in the plasma membrane when Yck is inactivated. Created by Emma Mohler.

Rgt2 levels were also measured in the akr1 deletion strain in figure 7. This strain interrupts the localization of Yck to the membrane, effectively removing any possible interaction with Rgt2. When Yck is delocalized, Rgt2 expression on the plasma membrane drops dramatically.

Figure 7

Rgt2 Expression in akr1 Knockout Strain



Note. WT+253 (WT) and akr1Δ+253 (akr1Δ) yeast were grown in SC-Leu+1% glucose to OD 1.3 and 1.0. Samples were grown in 2% glucose (+) or galactose (-) for 45 minutes. The plasma membrane was prepared per protocol. 12.5 ul of each sample were loaded into a 7.5% gel and run at 100 V. The primary antibody used for Western blot was HA antibody, and secondary was HRP antibody. When Yck is made unavailable to Rgt2 by removal from the plasma membrane, Rgt2 is not present on the cell surface. Created by Emma Mohler.

These three Western blots, all demonstrating the same expression pattern for Rgt2, indicate that Rgt2 relies on Yck to be stable on the plasma membrane.

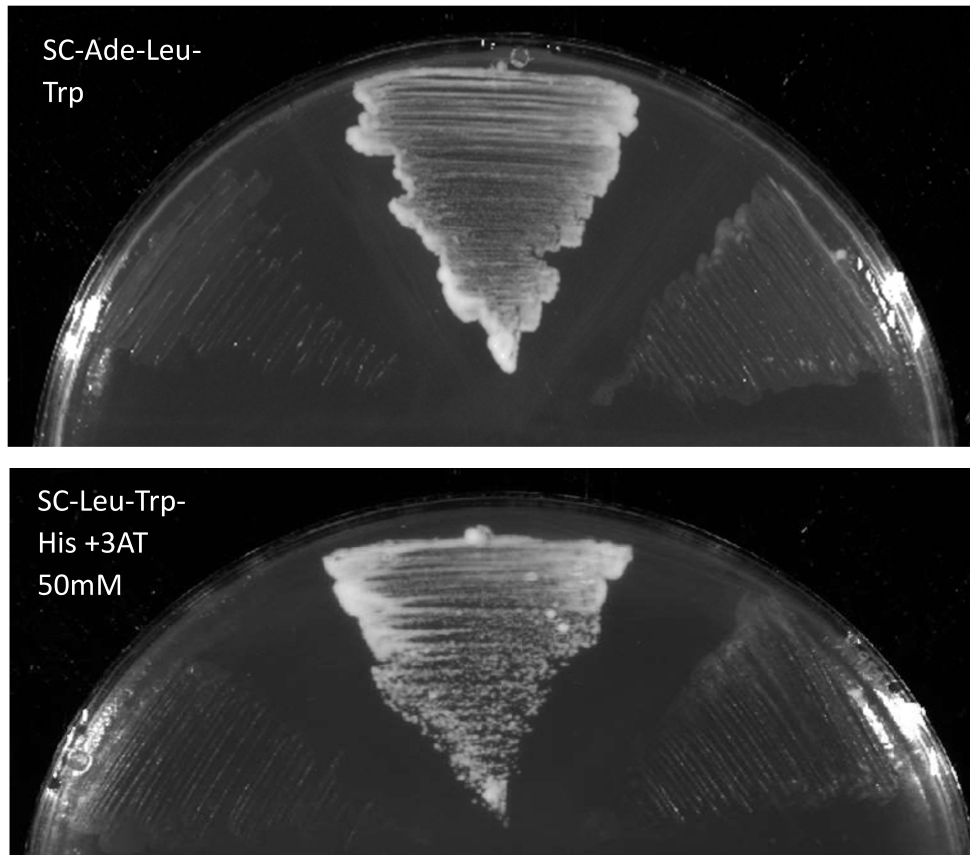
Yeast Two-Hybrid Assay Shows Yck Interacts with Key Phosphorylation Region

The results of the yeast two-hybrid assay between the Rgt2 C terminal domain and Yck1 are shown in figures 8 and 9. Yck1 and Rgt2 were shown to interact, as indicated by cell survival in figure 8. The deep blue color in figure 9 was a product of the activation of beta-galactosidase, resulting from the interaction of Yck1 and Rgt2. The key consensus phosphorylation sequence for the Ycks occurs in Rgt2 between amino acids 665-696. When amino acids 665-696 were removed, cell survival plummeted in figure 8 and the deep blue color faded to a very light blue in figure 9. Deleting these amino acids greatly reduced the measured interaction between Yck1 and

Rgt2. Because this sequence is well known for its potential for phosphorylation by the Ycks, this suggests that Yck phosphorylates Rgt2 at these amino acids.

Figure 8

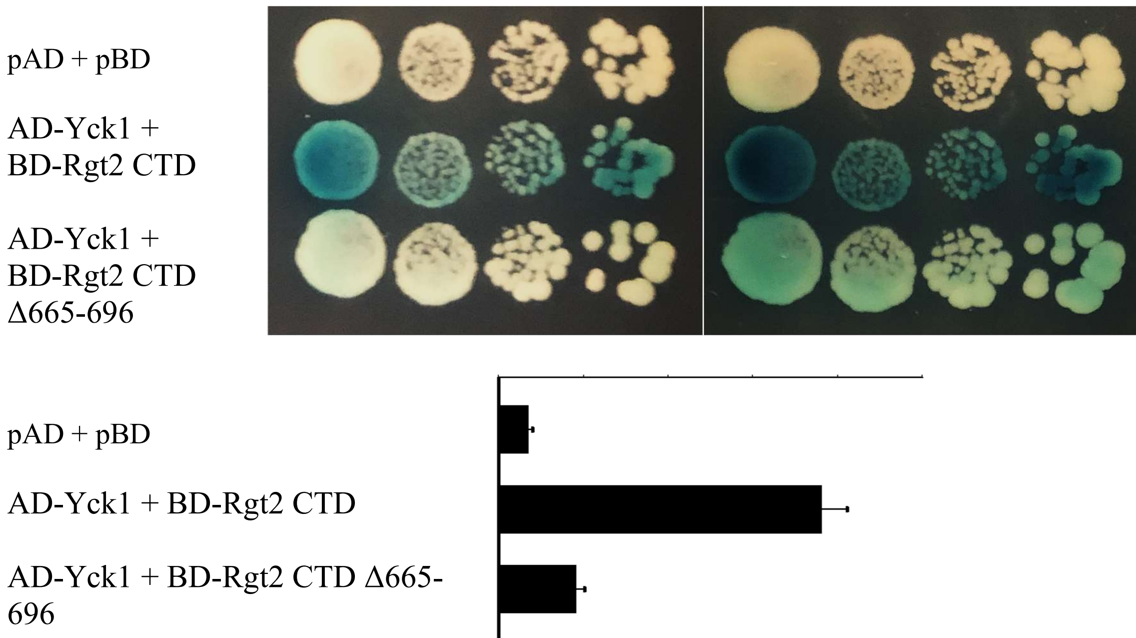
Yeast Two-Hybrid Assay with Rgt2 and Yck1 Shows Interaction



Note. Y2H yeast were transformed with three combinations of plasmids, plated from left to right: 1) AD/BD, 2) AD-Yck1 with BD-Rgt2, 3) AD-Yck1 with BD-Rgt2 Δ665-696. The transformed yeast were streaked onto selective plates where growth indicated interaction between Yck1 and Rgt2. Yck1 and Rgt2 are shown to interact strongly, but removing amino acids 665-696 decreases this interaction. Created by Emma Mohler.

Figure 9

Yeast Two-Hybrid Assay with Beta-Galactosidase Shows Interaction



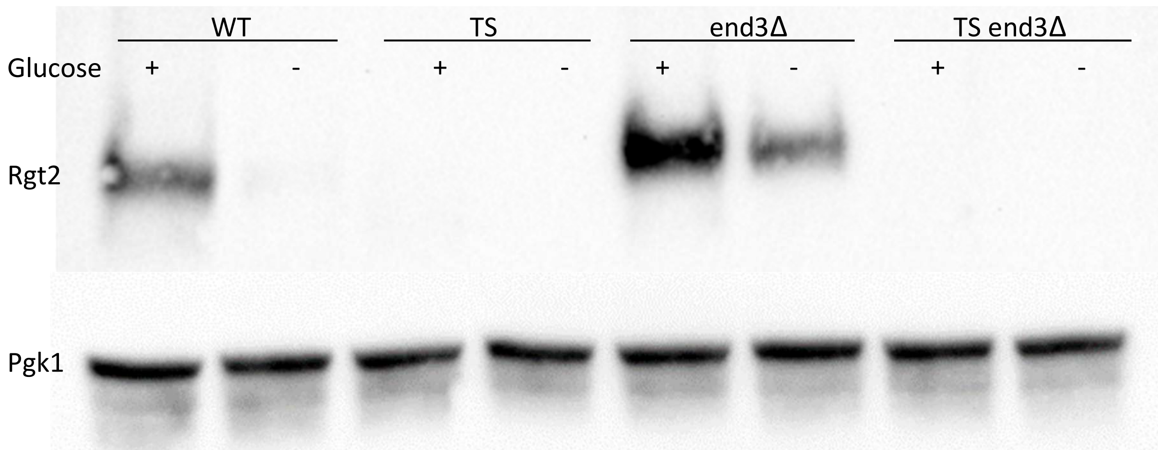
Note. The above transformants of the Y2H strain were plated on X-gal plates. The first picture was taken after 60 hours, and the second picture after 72 hours. The graph shows the beta-galactosidase assay. Yck1 and the Rgt2 CTD are demonstrated to interact strongly. Removing amino acids 665-696 greatly reduces this interaction. Created by Emma Mohler.

Yck Interaction with Rgt2 does not Control End3 Endocytosis

When the end3 endocytosis pathway is deactivated, Rgt2 is stable in the plasma membrane regardless of the presence of glucose. However, when this mutation is combined with the Yck temperature sensitive strain, Rgt2 is not present in the plasma membrane (Figure 10). This suggests that the role of Yck in stabilizing Rgt2 on the plasma membrane is not by controlling the progression of endocytosis by the end3 pathway.

Figure 10

Examination of Yck Deactivation with end3 Knockouts



Note. Yck WT+253, Yck TS+253, Yck WT end3Δ+253, and Yck TS end3Δ+253 yeast were grown to OD 1.4. The TS strains were then heat shocked at 37°C for 30 minutes. Samples were put in 2% glucose or galactose for 90 minutes. The plasma membrane was prepared per protocol. 20 ul of each sample was loaded into a 7.5% gel and run at 100 V. The primary antibody used for Western blot was HA antibody, and secondary was HRP antibody. Created by Emma Mohler.

Discussion

Yeast strongly prefer glucose as their source of energy. The complex system of glucose uptake and its flexibility for various extracellular glucose conditions attests to this. Rgt2 is a central component of the cellular response to high glucose conditions, and it allows the transcription of the glucose transporters appropriate for these conditions. It does this by interacting with the corepressors of the HXT genes, bringing Mth1 and Std1 in contact with the Ycks for phosphorylation and corresponding degradation. With this new evidence, it can also be concluded that the Ycks additionally phosphorylate Rgt2 for the purpose of increasing its stability on the plasma membrane in high glucose concentrations.

The Ycks often phosphorylate nutrient sensors to control their expression (Kim, Jeong-Ho et al., 2022). Usually, phosphorylation leads to the degradation of the nutrient sensors, in the presence of the nutrient that they sense. This evidence suggests that Rgt2 and the Ycks follow an opposite pattern: Rgt2 is phosphorylated to increase its stability, and it is kept stable on the plasma membrane when its nutrient is present in high concentrations. Rgt2 can then signal for the transporters necessary to bring in glucose. Rgt2’s continuing stability on the plasma membrane in high glucose concentrations ensures persistent transcription of the glucose transporters, and therefore guarantees that the cell can continue to import glucose.

It was originally expected that in the doubly mutated strain, Yck TS end3Δ, Rgt2 would continue to be expressed on the plasma membrane without respect to glucose concentration, because without end3 endocytosis, Rgt2 should have remained present. However, this was not found to be the case. Rgt2 is degraded in this strain. Therefore, the role of the Ycks in stabilizing Rgt2 on the cell surface is not by protecting it from endocytosis, but instead by some other means.

Future Work

The means by which the Ycks stabilize Rgt2 in glucose conditions on the plasma membrane is an area for future investigation. Additionally, this study focused specifically on Rgt2, which works in high glucose conditions. The connection between the Ycks and the stability of Snf3, the other glucose sensing receptor, is yet unexplored; more work can be done to see if similar connections exist as seen with Rgt2 and Yck, and studying Snf3 will expand these connections to low glucose conditions.

Conclusion

The glucose sensing receptor Rgt2 is stabilized by the yeast casein kinases in the presence of abundant glucose, triggering an intracellular signal for glucose transporters. This was demonstrated using Western blot in Yck deficient strains, showing a lack of stability of Rgt2 in glucose conditions when Yck was removed. Through a yeast two-hybrid experiment, Yck was also shown to interact with the C-terminal domain of Rgt2, specifically in the Yck phosphorylation consensus sequence found between amino acids 665-696. Interaction between Yck and Rgt2 specifically in the phosphorylation sequence strongly suggests phosphorylation. The Ycks do not specifically protect Rgt2 from end3 endocytosis but stabilize it by another means. This study yields new insights into the process of glucose uptake and regulation in yeast, as well as the casein kinases.

References

Busti, S., Coccetti, P., Alberghina, L., & Vanoni, M. (2010). Glucose signaling-mediated coordination of cell growth and cell cycle in saccharomyces cerevisiae; 22219709. *Sensors, 10*(6), 6195-6240. doi:10.3390/s100606195

Flick, K. M., Spielewoy, N., Kalashnikova, T. I., Guaderrama, M., Zhu, Q., Chang, H. -., & Wittenberg, C. (2003). Grr1-dependent inactivation of Mth1 mediates glucose-induced dissociation of Rgt1 from HXT gene promoters; 12925759. *Molecular Biology of the Cell, 14*(8), 3230-3241. doi:10.1091/mbc.E03-03-0135

Hantzidiamantis, P. J., & Lappin, S. L. (2021). *Physiology, glucose*. Florida: StatPearls Publishing.

Kim, J. -., & Johnston, M. (2006). Two glucose-sensing pathways converge on Rgt1 to regulate expression of glucose transporter genes in saccharomyces cerevisiae; 16844691. *Journal of Biological Chemistry, 281*(36), 26144-26149. doi:10.1074/jbc.M603636200

Kim, J. -., Polish, J., & Johnston, M. (2003). Specificity and regulation of DNA binding by the yeast glucose transporter gene repressor Rgt1; 12861007. *Molecular and Cellular Biology, 23*(15), 5208-5216. doi:10.1128/MCB.23.15.5208-5216.2003

Kim, J., Bloor, D., Rodriguez, R., Mohler, E., Mailloux, L., Melton, S., & Jung, D. (2022). Casein kinases are required for the stability of the glucose-sensing receptor Rgt2 in yeast. *Scientific Reports, 12*(1) doi:10.1038/s41598-022-05569-1

Kim, J., Brachet, V., Moriya, H., & Johnston, M. (2006). Integration of transcriptional and posttranslational regulation in a glucose signal transduction pathway in *saccharomyces cerevisiae*. *Eukaryot Cell, 5*(1), 167. doi:10.1128/EC.5.1.167-173.2006

Kim, J., & Rodriguez, R. (2021). Glucose regulation of the paralogous glucose sensing receptors Rgt2 and Snf3 of the yeast saccharomyces cerevisiae. *Biochimica Et Biophysica Acta (BBA) - General Subjects, 1865*(6), 129881. doi:https://doi.org/10.1016/j.bbagen.2021.129881

Kim, J., Roy, A., Jouandot, D., & Cho, K. H. (2013). The glucose signaling network in yeast. *Biochimica Et Biophysica Acta (BBA) - General Subjects, 1830*(11), 5204-5210. doi:https://doi.org/10.1016/j.bbagen.2013.07.025

Lafuente, J., Gancedo, C., Jauniaux, J., & Gancedo, J. M. (2000). Mth1 receives the signal given by the glucose sensors Snf3 and Rgt2 in saccharomyces cerevisiae. *Molecular Microbiology, 35*(1), 161-172.

Lakshmanan, J., Mosley, A. L., & Özcan, S. (2003). Repression of transcription by Rgt1 in the absence of glucose requires Std1 and Mth1; 14508605. *Current Genetics, 44*(1), 19-25. doi:10.1007/s00294-003-0423-2

Moriya, H., & Johnston, M. (2004). Glucose sensing and signaling in saccharomyces cerevisiae through the Rgt2 glucose sensor and casein kinase I; 14755054. *Proceedings of the National Academy of Sciences of the United States of America, 101*(6), 1572-1577. doi:10.1073/pnas.0305901101

Özcan, S., Dover, J., Rosenwald, A. G., Wölfl, S., & Johnston, M. (1996). Two glucose transporters in saccharomyces cerevisiae are glucose sensors that generate a signal for induction of gene expression; 8901598. *Proceedings of the National Academy of Sciences of the United States of America, 93*(22), 12428-12432. doi:10.1073/pnas.93.22.12428

Ozcan, S., & Johnston, M. (1995). Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. *Molecular and Cellular Biology, 15*(3), 1564-1572. doi:10.1128/MCB.15.3.1564

Özcan, S., & Johnston, M. (1999). Function and regulation of yeast hexose transporters. *Microbiology and Molecular Biology Reviews, 63*(3), 554. doi:10.1128/MMBR.63.3.554-569.1999

Pasula, S., Chakraborty, S., Choi, J. H., & Kim, J. (2010). Role of casein kinase 1 in the glucose sensor-mediated signaling pathway in yeast. *BMC Cell Biology, 11*(1), 17. doi:10.1186/1471-2121-11-17

Rolland, F., Winderickx, J., & Thevelein, J. M. (2001). Glucose-sensing mechanisms in eukaryotic cells. *Trends in Biochemical Sciences, 26*(5), 310-317. doi:https://doi.org/10.1016/S0968-0004(01)01805-9

Scharff-Poulsen, P., Moriya, H., & Johnston, M. (2018). Genetic analysis of signal generation by the Rgt2 glucose sensor of saccharomyces cerevisiae. *G3 Genes|Genomes|Genetics, 8*(8), 2685-2696. doi:10.1534/g3.118.200338

Schmidt, M. C., McCartney, R. R., Zhang, X., Tillman, T. S., Solimeo, H., Wölfl, S., . . . Watkins, S. C. (1999). Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in saccharomyces cerevisiae; 10373505. *Molecular and Cellular Biology, 19*(7), 4561-4571. doi:10.1128/MCB.19.7.4561

Snowdon, C., & Johnston, M. (2016). A novel role for yeast casein kinases in glucose sensing and signaling. *Molecular Biology of the Cell, 27*(21), 3369-3375. doi:10.1091/mbc.E16-05-0342

Towle, H. C. (2005). Glucose as a regulator of eukaryotic gene transcription. *Trends in Endocrinology & Metabolism, 16*(10), 489-494. doi:https://doi.org/10.1016/j.tem.2005.10.003