Coordinated regulation of intracellular pH by two glucose-sensing pathways in yeast

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The yeast *Saccharomyces cerevisiae* employs multiple pathways to coordinate sugar availability and metabolism. Glucose and other sugars are detected by a G protein–coupled receptor, Gpr1, as well as a pair of transporter-like proteins, Rgt2 and Snf3. When glucose is limiting, however, an ATP-driven proton pump (Pma1) is inactivated, leading to a marked decrease in cytoplasmic pH. Here we determine the relative contribution of the two sugar-sensing pathways to pH regulation. Whereas cytoplasmic pH is strongly dependent on glucose abundance and is regulated by both glucose-sensing pathways, ATP is largely unaffected and therefore cannot account for the changes in Pma1 activity. These data suggest that the pH is a second messenger of the glucose-sensing pathways. We show further that different sugars differ in their ability to control cellular acidification, in the manner of inverse agonists. We conclude that the sugar-sensing pathways act via Pma1 to invoke coordinated changes in cellular pH and metabolism. More broadly, our findings support the emerging view that cellular systems have evolved the use of pH signals as a means of adapting to environmental stresses such as those caused by hypoxia, ischemia, and diabetes.

Nature provides a variety of sugars of varying complexity and abundance. Like most organisms, yeast preferentially use glucose as a source of carbon and energy, and there are multiple signaling pathways that respond accordingly. When preferred sugars are in short supply, the resulting changes in metabolism are mediated by the protein kinase Snf1 (sucrose non-fermenting 1), the founding member of the AMP-activated protein kinase (AMPK)[3] family (1, 2).

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[3] The abbreviations used are: AMPK, AMP-activated protein kinase; PKA, cAMP-dependent protein kinase; PDB, Protein Data Bank; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; fQCR, fast quantitative cysteine reactivity; SC, synthetic complete.

Two other protein kinase systems, considered here, coordinate the response to glucose availability and thereby act in opposition to Snf1. Of these, the best understood is the cAMP-dependent protein kinase (PKA). PKA is activated by cAMP, a second messenger generated from ATP by the adenylyl cyclase enzyme Cyr1 (3–6). The addition of glucose to starved cells results in a 2–3-fold spike of cAMP (7–9). Upon cAMP binding to the PKA regulatory subunit (Bcy1), the catalytic subunits (Tpk1, Tpk2, and Tpk3) are liberated, and each phosphorylates a distinct panel of substrates (10–16).

The enzyme adenylyl cyclase is regulated by both large and small (Ras family) G proteins (5, 9, 17–26). The large G protein is composed of a typical Gα protein, Gpa2, and an atypical Gβ subunit, Asc1 (27). Gpa2 is activated by the cell surface receptor Gpr1 (7, 28) (29–32) and inactivated by the GTPase-accelerating protein Rgs2 (33). The small G proteins Ras1 and Ras2 are activated by the guanine nucleotide exchange factors Cdc25 and Sdc25 (34–40) and inactivated by Ira1 and Ira2 (41–44). It is not known how Cdc25 and Sdc25 are themselves activated.

Another glucose-sensing system consists of Yck1 and Yck2 (yeast type I casein kinase). These kinases are physically associated with the cell surface proteins Rgt2 and Snf3. Although they are homologous to glucose transporters, Rgt2 and Snf3 appear to have lost their transporter function and instead serve exclusively as receptor or "transceptor" proteins. Following glucose addition (45–47), Rgt2 and Snf3 recruit the transcription corepressors Mth1 and Std1 (48, 49), which are then phosphorylated by Yck1 and Yck2, ubiquitinated, and degraded (50–52). The destruction of Mth1 and Std1 derepresses genes encoding hexose transporters and promotes the uptake of the newly available sugars (47, 53–62).

Whereas much is known about the various glucose-sensing pathways, comparatively little is known about how their activities are coordinated. One way that cells can integrate signaling is through the production of chemical second messengers. For example, glucose abundance stimulates adenylyl cyclase activity, cAMP production, and activation of PKA (reviewed in Ref. 63). Conversely, glucose limitation leads to increased cellular ADP and activation of AMPK (64, 65). Thus, there is a reciprocal regulation of two adenosine metabolites, cAMP and ADP, and two distinct kinases, PKA and AMPK. These kinases then converge on at least one major transcription factor, Msn2. Whereas AMPK/Snf1 phosphorylates and activates Msn2 (66,

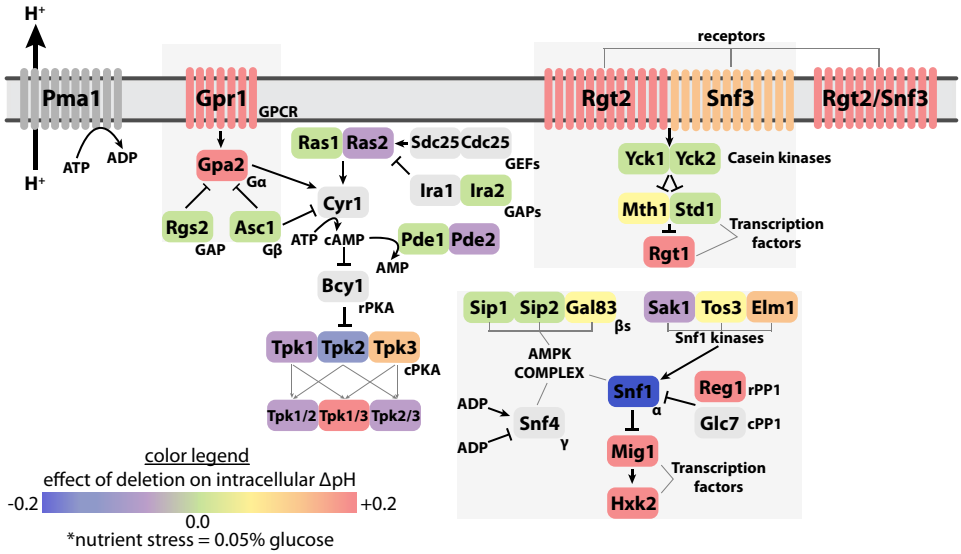

Figure 1. Sugar-sensing pathways couple sugar stress to intracellular acidification. Three pathways in *S. cerevisiae* respond to changes in sugar availability. *Colors* of signaling components indicate the observed effects of deletion mutations on intracellular pH, as described under "Results." *Key*, ranked *color groupings* correspond to a mean $\Delta pH \leq -0.2$ (*dark blue*), $-0.2 > \Delta pH \leq -0.1$ (*blue*), $-0.1 > \Delta pH \leq -0.05$ (*purple*), $-0.05 > \Delta pH < 0.05$ (*green*), $0.05 \geq \Delta pH < 0.1$ (*yellow*), $0.1 \geq \Delta pH < 0.2$ (*orange*), and $\Delta pH \geq 0.2$ (*red*). Components required for proper growth or viability were not tested (*gray*). *GEF*, guanine nucleotide exchange factor; *GAP*, GTPase activating protein; *rPKA* and *cPKA*, regulatory and catalytic subunits of protein kinase A; *rPP1* and *cPP1*, regulatory and catalytic subunits of the protein phosphatase.

67), PKA phosphorylation prevents Msn2 from translocating to the nucleus (68–70). There is also an additional layer of regulation wherein Snf1 phosphorylates and inhibits adenylyl cyclase and the PKA pathway (71) and, conversely, PKA phosphorylation inhibits the Snf1 response (72). These findings suggest the existence of a mutual inhibitory network that acts in response to glucose limitation and that can generate distinct dynamic patterns of transcription factor activation (73).

Here we consider the role of pH as a potential second messenger of glucose availability. It is well-established, but perhaps not widely appreciated, that cytoplasmic pH drops by as much as a full log unit in glucose-limiting conditions (74–76). Much of this change is probably due to inactivation of Pma1, an ATP-driven proton pump and master regulator of yeast intracellular pH that is located at the plasma membrane (77). Our approach was to systematically delete components of the two known glucose-sensing pathways (Rgt2/Snf3 and Gpr1) and quantify cellular pH, over time, at high or low concentrations of a variety of sugar sources. We find that both pathways regulate cellular acidification and do so in a coordinated manner. Finally, we show that naturally abundant sugars inhibit cellular acidification and do so in the manner of pharmacological inverse agonists.

Results

Intracellular acidification in response to glucose stress

Glucose starvation is well-known to promote the induction of stress-responsive genes (78). Also well-documented, but not widely appreciated, is the ability of glucose to regulate the pH of the cytoplasm; when glucose is limiting, intracellular pH drops by as much as a full log unit (74–76). However, the cause and consequences of these changes have remained obscure. To address this gap in our understanding, we tested the hypothesis that pH serves as a second messenger for one or more of the glucose-sensing pathways in yeast (Fig. 1).

We began by comparing 45 different gene deletion strains, each lacking one or more core components of the glucose pathways. These deletions represent receptors, effectors, protein kinases, and transcription factors (Fig. 1). For several closely related protein kinases and receptors, we also analyzed double and triple deletion mutants. We did not consider genes essential for viability or that exhibit severe growth defects when absent: Pma1 (ATP-driven proton pump), Cyr1 (adenylyl cyclase), Bcy1 (PKA regulatory subunit), Snf4 (AMPK regulatory subunit), Glc7 (AMPK phosphatase), Ira1 (Ras GTPase–activating protein), and Sdc25 and Cdc25 (activators of Ras). To detect dynamic changes in cellular pH, we transformed each strain with pHluorin, a ratiometric pH reporter based on the green fluorescent protein (75, 79).

Cells were grown to mid-log phase in the presence of 1.6% (w/v) glucose (hereafter "high" glucose), washed, and resuspended in medium containing either no glucose or one of seven different glucose concentrations ranging from 0.025 to 1.6%. As illustrated in Fig. 2*A* for wildtype yeast, pH measurements were made immediately after resuspending the cells and then monitored for 30 min in a microplate reader. Kinetic pH traces were collected for each deletion strain. These pH traces were nearly identical (within 0.1 pH units of wildtype) at higher glucose concentrations (>0.1%) but differed in several cases at lower glucose concentrations. This result is illustrated in Fig. 2*B* at a single time point (10 min) for wildtype and two representative strains missing glucose-sensing pathway components (*gpa2*Δ and *rgt2*Δ). Based on this finding, we refined our approach to focus on pH responses at lower glucose concentrations. We proceeded by quantifying intracellular pH in the 45 deletion strains at a single time point (10 min) after treatment with a single glucose concentration that evokes a nutrient stress response (0.05%) (Fig. 2*C*) (63).

As is shown in Fig. 2*C*, a continuum of intracellular pH values was observed in response to glucose limitation. It is notable that

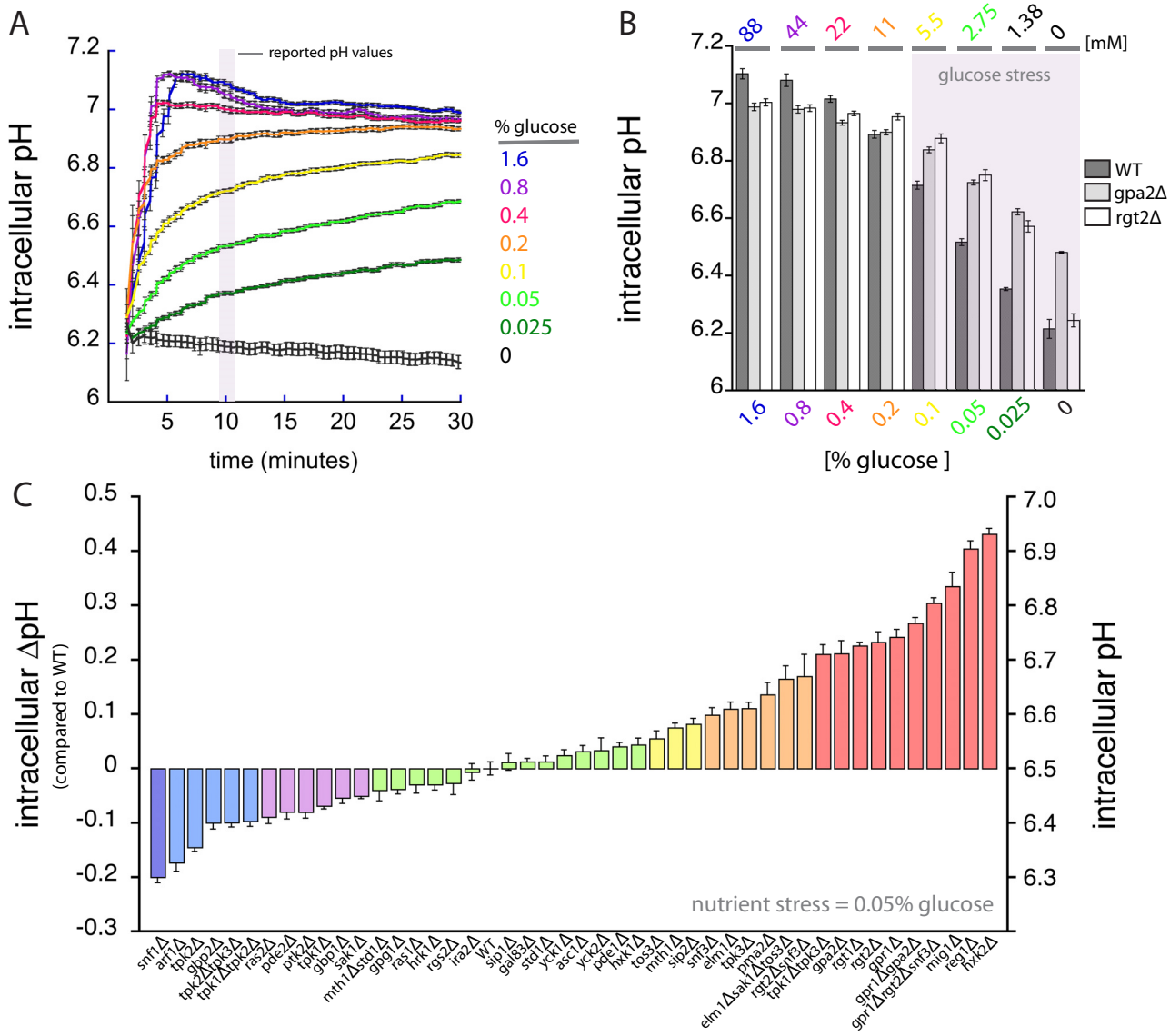

Figure 2. Intracellular acidification in response to glucose stress. Intracellular pH measurements as reported by the genetically encoded pH biosensor pHuorin. *A*, kinetics of intracellular pH recovery in response to glucose readdition in wildtype yeast. Cells were grown in 2% glucose to $A_{600\,nm}$ ~1.0, harvested by centrifugation, and washed twice in glucose-free medium before adding the indicated concentration of glucose. Equivalent pH recovery profiles were measured for each strain in this study. pH values for each of the glucose concentrations were compared at the 10-min time internal within each kinetic trace (indicated by the *vertical gray band*). *B*, intracellular pH recovery 10 min after glucose readdition for WT and yeast strains missing two exemplary components of sugar-sensing pathways, a G-protein α subunit (Gpa2) and sugar receptor (Rgt2). The *shaded gray area* indicates the operational definition of stress-inducing concentrations of glucose. *C*, intracellular pH recovery 10 min after the readdition of 0.05% glucose in yeast strains missing one or more sugar-sensing pathway components. pH values are ranked from *left* to *right* by $\Delta pH = pH_{variant\ strain} - pH_{WT\ strain}$. In each case, intracellular pH is compared with wildtype. Ranked *color groupings* correspond to those shown in Fig. 1 (*key*). *Error bars* in *A*, S.D. of $n = 12$ independent kinetic experiments. *Error bars* in *B* and *C*, S.E. for at least $n = 3$ independent experiments.

these pH differences would have been missed if only higher glucose concentrations had been studied. As the data in Fig. 2*C* indicate, most deletion strains exhibited a change in pH similar (within 0.2 units) to wildtype cells. In contrast, deletion of the AMPK gene (*snf1*Δ) resulted in overacidification by >0.2 pH units. Conversely, and unexpectedly, many of the other deletion strains exhibited a dramatic underacidification (>0.2 pH units); these include cells lacking putative glucose receptors (*rgt2*Δ or *gpr1*Δ), the Gα protein (*gpa2*Δ), the AMPK phosphatase adaptor protein (*reg1*Δ), and several transcription factors (*mig1*Δ, *hxk2*Δ, and *rgt1*Δ). The acidification defect was greater in the combined absence of *GPR1* and *RGT2*/*SNF3* than in the absence of either pathway alone (Fig. 2*C*). These results indicate that the known glucose signaling systems work in parallel to regulate cytoplasmic pH (*i.e.* pH is a general readout of glucose availability). Whereas sensors of glucose abundance (Rgt2/Snf3 or Gpr1) are necessary for proper cellular acidification, the intracellular sensor of glucose limitation (Snf1) is needed to prevent overacidification. We conclude that the Rgt2/Snf3 and Gpr1 pathways act in a non-redundant and coordinated manner.

Regulation of intracellular acidosis in response to sugar stress is largely independent of ATP levels

Glucose could function in one of two ways to maintain normal pH in the cell. First, glucose could function pharmacologically (*e.g.* by binding to cell surface receptors). Given the high

effective concentrations of glucose necessary to evoke a response, it is not currently feasible to conduct standard equilibrium-based interaction assays for these ligands or their receptors. Thus, it is conceivable that the receptors do not bind directly to sugars, or these sugars function as allosteric regulators rather than orthosteric ligands. Alternatively, glucose metabolism could produce one or more products that activate protein targets within the cell (80). For example, glucose drives the production of ATP, which is needed to fuel the extrusion of metabolic proton equivalents by the ATP-driven proton pump, Pma1. ATP is also an essential metabolic precursor of cAMP, which binds to and activates PKA. Thus, it is plausible that cAMP is produced via mass action merely as a result of increased ATP production.

To better understand the mechanisms by which glucose levels regulate intracellular pH, we sought to determine the correspondence between pH, ATP (which activates Pma1 and adenylyl cyclase), and cAMP (which activates PKA). Our goal was to quantify relative intracellular ATP, cAMP, and glucose levels from single experimental samples collected under conditions of high and low glucose. To this end, we turned to mass spectrometry, which is widely considered the most reliable method for measuring the relative abundance of metabolites in complex mixtures. Given the pleiotropic effects of the AMPK pathway, we restricted our analysis to the Rgt2/Snf3 and Gpr1/Gpa2 signaling axes. Wildtype and select deletion strains (*rgt2*Δ, *snf3*Δ, *gpr1*Δ, and *gpa2*Δ) were washed and resuspended in either 1.6 or 0.05% glucose for 10 min. Soluble cell lysates were then spiked with isotope-labeled ATP and AMP and then quantified by HPLC-MS/MS analysis. Glucose was measured as an internal reference control, and data were collected as the peak area ratio for each metabolite and its corresponding stable isotope standard. Stable isotope-labeled ATP was used for analyte ATP; stable isotope-labeled AMP was used for analyte cAMP and glucose.

As shown in Fig. 3*A* and Table S1 and as reported previously using other methods (81), intracellular ATP levels remained high in glucose-limiting conditions. ATP levels were similarly maintained (or elevated) in the four deletion strains (*rgt2*Δ, *snf3*Δ, *gpr1*Δ, and *gpa2*Δ). Thus, it appears that the accumulation of intracellular protons is not the result of Pma1 inactivation caused by metabolic depletion of ATP. Rather, our data support an alternative hypothesis wherein Pma1 is inactivated before any depletion of ATP, possibly to preserve ATP for other essential metabolic activities. As shown in Fig. 3*B*, intracellular cAMP levels were also unaffected by changes in glucose abundance (confirmed experimentally; Fig. 3*C*). In light of these observations, we conclude that adenosine nucleotide metabolism and pH are regulated independently. Whereas the known glucose receptors are critically important for proper pH regulation, they are not required to maintain high cellular levels of ATP or cAMP.

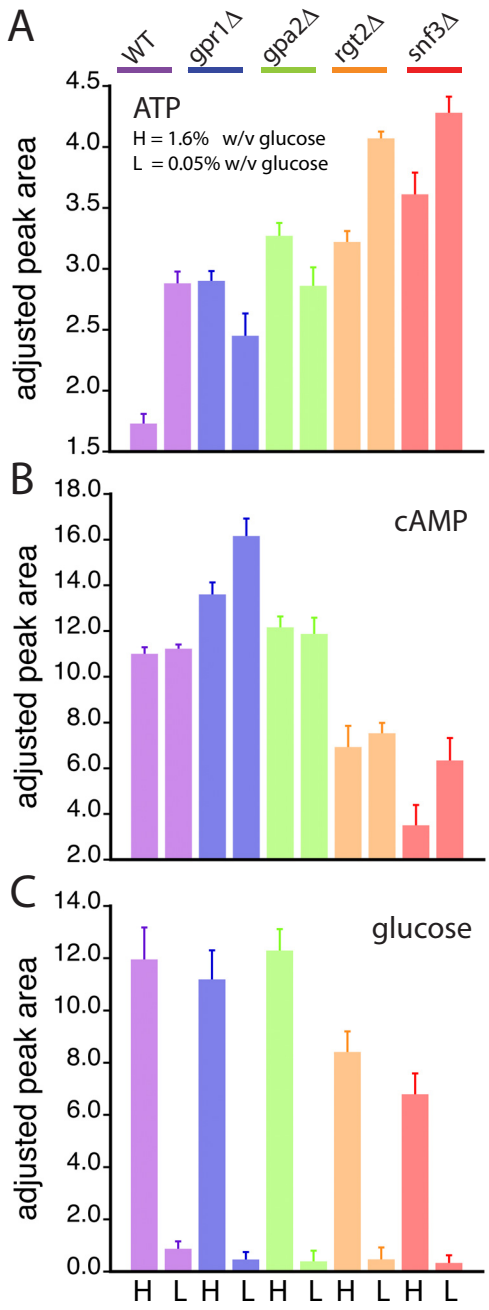

Figure 3. Intracellular acidification in response to glucose stress is largely independent of ATP and cAMP levels. *A–C*, intracellular ATP (*A*), cAMP (*B*), and glucose (*C*) simultaneously measured by LC-MS. Cells were grown in 2% (w/v) glucose to $A_{600\ nm}$ = 1.0, harvested by centrifugation, and washed with glucose-free medium before a 10-min treatment with high (*H* = 1.6%) or low (*L* = 0.05%) glucose. *Bar height* is the average of 4–6 measurements and represents the adjusted peak area for each compound. Adjusted peak area for each compound was calculated by dividing the peak area for that compound by the peak area for the stable isotope-labeled ATP or AMP standard. *Error bars*, S.E. for at least *n* = 3 independent experiments.

Gpa2 is both a pH sensor and sugar-sensing pathway component

Several previous observations led us to reason that the G protein α subunit, Gpa2, is likely to be a sensor of intracellular pH. Prior structural informatics calculations by our group have shown that buried ionizable networks are a structural hallmark of Gα pH sensitivity (82). Using a variety of biophysical techniques, we and others have shown that pH regulates the conformation of G protein α subunits, both in yeast (Gpa1) and animal cells ($G\alpha_i$) (83, 84). However, we know from this work that we cannot mutate residues that are predicted to account for pH-sensing activity (82). Mutations within this highly con-

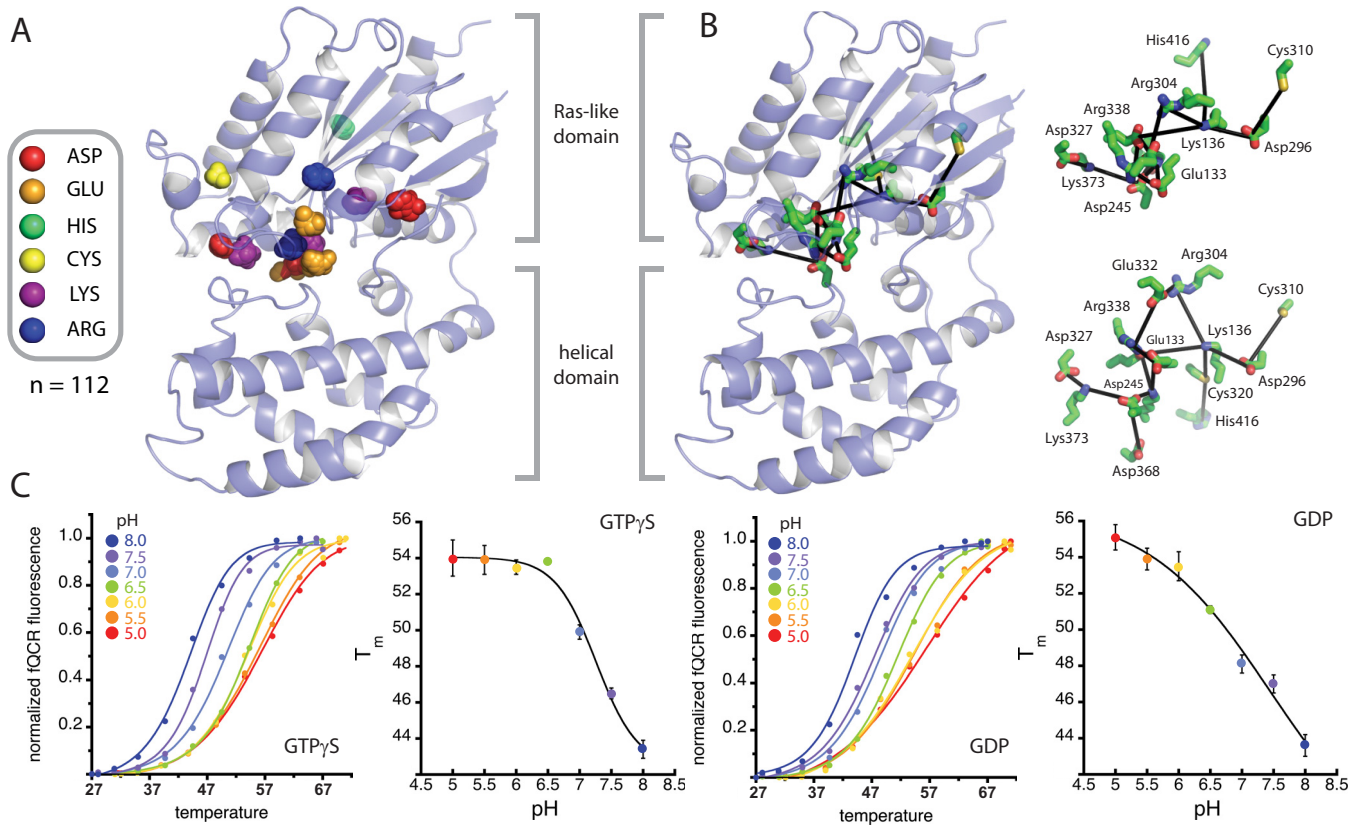

Figure 4. The Gα subunit Gpa2 is both a pH sensor and sugar-sensing pathway component. *A*, spatial conservation of buried ionizable side chains within 112 G protein α subunit crystal structures calculated using pHinder. In Gα subunits, most of these conserved ionizable side chains sample the interface between the Ras-like and helical domains. *B*, a homology model of the yeast Gα subunit, Gpa2, and its predicted network of buried ionizable side chains. *C*, pH-dependent fQCR profiles for Gpa2 (overexpressed and purified from *E. coli*) in the presence of GTP analog (GTPγS) and GDP. fQCR profiles at each pH value represent an average of three independent thermal unfolding experiments. The *solid line* through the data points of each fQCR profile represents a linear least-squares fit of the Gibbs–Helmholtz equation, which was used to quantify the midpoint of thermal denaturation (T_m) of each fQCR profile at each pH value. The *solid line* through the T_m *versus* pH profiles represents a modified Hill equation. *Error bars*, S.E. for $n = 3$ independently measured T_m values.

served network (*e.g.* Lys-270 and Lys-277 of Gα_i) failed to express and could not be analyzed biochemically (82).

In Fig. 4*A*, we provide an updated informatics analysis, using our newer approach known as consensus network analysis (82). This calculation shows that buried ionizable networks are spatially conserved in the full set of 112 Gα protein structures available in the Protein Data Bank (PDB). Furthermore, this pH-sensitive network is also conserved in the homology model of yeast Gpa2 shown in Fig. 4*B*. Together, these observations suggest that Gpa2 is also regulated by changes in intracellular pH.

To test whether Gpa2 structure and function is pH-regulated, we overexpressed and purified the protein from bacteria and measured its thermostability as a function of pH. As with GTPγS-bound Gpa1 and Gαi (83, 84), Gpa2 exhibited a highly cooperative pH-dependent shift in thermostability (T_m value) (Fig. 4*C*), ranging from a T_m value of 54 °C at pH 5.0 to a T_m value of 44 °C at pH 8.0. These observations indicate that Gpa2 has the structural and functional hallmarks of a pH-sensing protein. In contrast to other Gα proteins, however (82), the effects of pH are similar for the GDP- and GTPγS-bound forms of the protein. We conclude that Gpa2 undergoes pH-dependent conformational changes in both the activated and inactivated states.

Sugar sensors and G proteins couple carbon stress to intracellular acidity

As shown above, we could not detect appreciable changes in intracellular ATP or cAMP levels as a function of glucose concentration or in the absence or presence of glucose receptors. This is in marked contrast to the dramatic changes in cytoplasmic pH that we observed under the same experimental conditions. Moreover, because deletion of components in different sugar-sensing pathways has similar effects on cytoplasmic pH, we inferred that Gpr1/Gpa2 and Rgt2/Snf3 have non-redundant signaling functions. Thus, we considered an alternative model where glucose is not only a nutrient but also a ligand that dictates the abundance of a proton second messenger. Given that receptors are necessary for proper acidification, and glucose prevents acidification, we surmised that glucose functions as an "inverse agonist" with respect to pH signaling (*i.e.* binding of glucose puts the receptor in an inactivated state and prevents receptor-mediated cellular acidification). By extension, other sugars might behave as agonists or partial agonists (or lack activity entirely and are simply metabolized).

As an initial test of the model, we measured cellular pH in response to a panel of sugars, at high or low concentrations, and in the presence or absence of select signaling components (Rgt2, Snf3, Gpr1, and Gpa2). As shown in Fig. 5*A*, intracellular

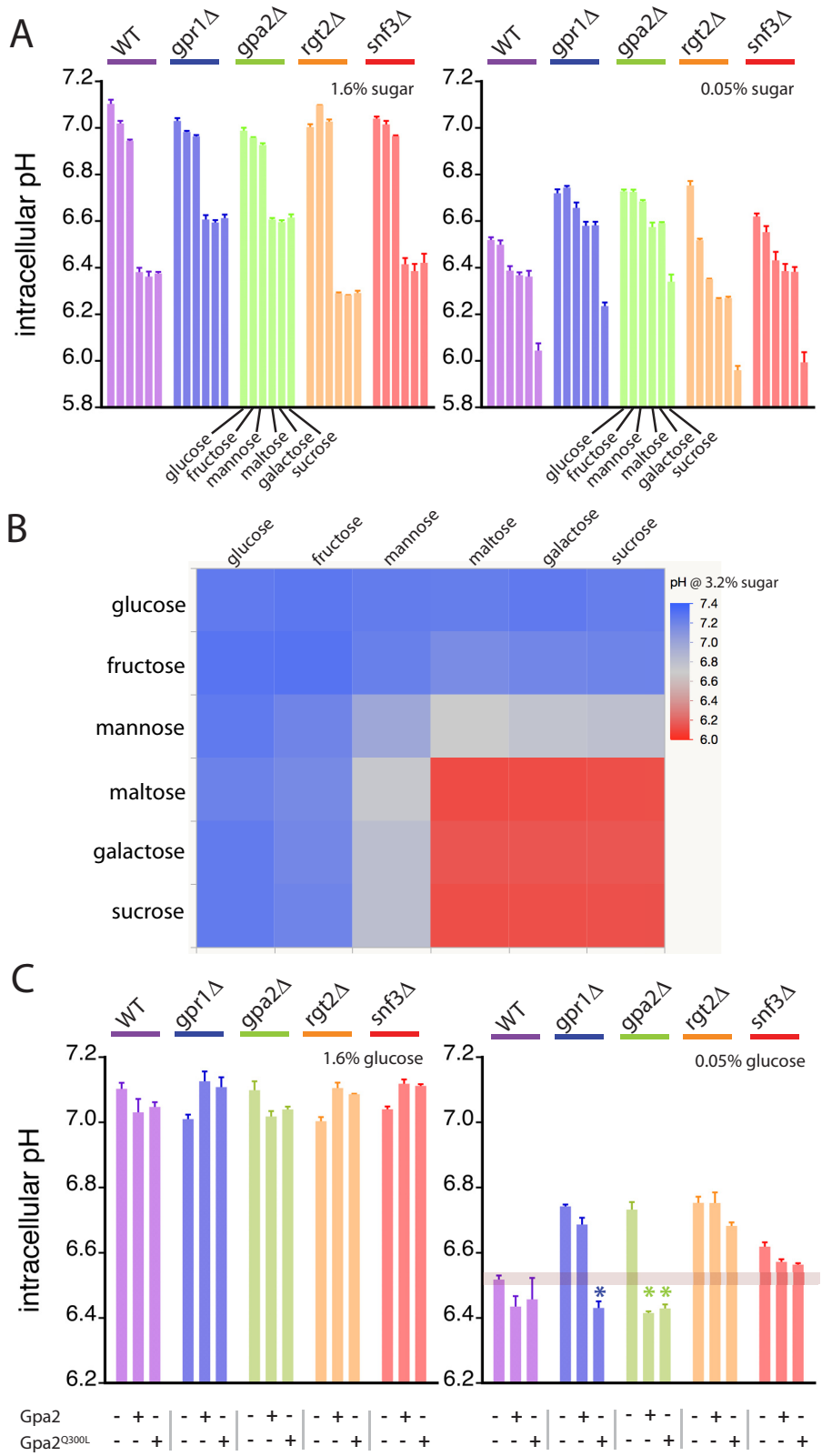

Figure 5. Sugar sensors and G proteins couple carbon stress to intracellular acidification. *A*, intracellular pH recovery 10 min after the readdition of high (1.6%) or low (0.05%) sugar for WT and yeast strains missing individual sugar receptors (Gpr1, Rgt2, and Snf3) or the G-protein α subunit (Gpa2). *B*, intracellular pH recovery of WT yeast 10 min after the readdition of pairwise sugar mixtures. Each element of the pairwise sugar-mixing matrix contained a total of 3.2% sugar (*i.e.* 1.6% of each combined sugar). Reported pH values correspond to an average of three independent experiments originating from three different yeast colonies. *C*, the effects of episomal overexpression of Gpa2 or a constitutively active Gpa2 variant ($Gpa2^{Q300L}$) on pH recovery 10 min after the readdition of high or low glucose. *Asterisks*, conditions under which Gpa2 overexpression (*green*) and constitutive activity (*blue*) rescue the pH recovery phenotype of WT at 0.05% glucose (indicated by the *horizontal gray band*). *Error bars* in *A* and *C*, S.E. for at least $n = 3$ independent experiments. pH values and S.E. values for *B* are available in Table S4.

pH was within the normal range when maintained in high concentrations (1.6%) of glucose, fructose, and mannose. In contrast, intracellular pH was substantially lower when maintained in low concentrations of these same sugars (Fig. 5*A*). Thus, according to our model, glucose, fructose, and mannose serve as inverse agonists (or negative allosteric regulators) of both sugar-sensing pathways. Other sugars (maltose, sucrose, and galactose) appear to lack any signaling activity. Indeed, the readdition of these three other sugars, in a pairwise fashion and at high concentration (1.6%), does not affect the apparent inverse agonism of fructose, glucose, and mannose (Fig. 5*B*). Moreover, any metabolism of disaccharides to glucose and fructose is evidently insufficient to affect signaling over the duration of the experiment (10 min) (85). From these observations, we conclude that Rgt2/Snf3 and Gpr1/Gpa2 maintain intracellular acidosis (the default state) in the absence of inhibitory sugar ligands.

Last, we assessed the relative importance of Gpa2 pH sensing in both sugar-sensing pathways (Fig. 5*C*). To this end, we measured intracellular pH under conditions of high and low glucose, in wildtype and select deletion strains transformed with a constitutively active (GTPase-deficient) Gα mutant, Gpa2^{Q300L} (Fig. 5*C*) (8). As expected, constitutive Gpa2 activity restored the acidotic state under conditions of low glucose in the *gpr1*Δ and *gpa2*Δ strains. Furthermore, these data indicate that loss of Gpr1 inhibition under conditions of low glucose is required for Gpa2 activation, which mirrors the establishment of intracellular acidification. From these observations, we conclude that Rgt2/Snf3 and Gpr1 (via Gpa2-GTP) work in concert to maintain intracellular acidosis (the default state) except when prevented from doing so by an inhibitory sugar ligand (glucose, fructose, and mannose).

Discussion

Here we present several new and important features of the glucose-sensing apparatus in yeast. We show that multiple sugars and sugar-sensing pathways regulate cellular pH. Whereas AMPK prevents cellular acidification, activation of the PKA and YCK phosphorylation cascades promotes increased acidification. Adenosine metabolites are, comparatively speaking, unaffected. Moreover, a key upstream component of the PKA pathway (Gpa2) is itself sensitive to pH. Given that changes in pH are a common feature of all three sensing pathways, are affected by a variety of different sugars, and are detected by a key component of one of the pathways, we propose that changes in proton abundance help to coordinate cellular responses to nutrient availability.

Our initial hypothesis was that a reduction in ATP availability might account for the reduction in cellular pH. This hypothesis was based on the expectation that glucose limitation would slow glycolysis and reduce intracellular ATP levels. This, in turn, would diminish proton extrusion from the cytoplasm by Pma1, the ATP-driven proton pump that serves as the master regulator of yeast intracellular pH. On the other hand, at least one other group, using an alternative method, has shown that ATP levels *increase* in response to glucose limitation in yeast (81). Thus, a reduction in glucose may instead lead to the direct inactivation of Pma1 and other ATP-driven processes, possibly as a way to conserve energy in the face of looming starvation.

Apart from any effect of ATP, the changes in pH are due, at least in part, to the phosphorylation of Pma1. It is well-established that glucose addition leads to robust (10-fold), rapid (<10 min), and reversible phosphorylation and activation of Pma1 (77, 86, 87). Although the protein kinase(s) have not been identified, it seems likely that Pma1 is targeted by protein kinases downstream of Gpr1 and/or Snf3/Rgt2. In support of this model, deletion of either *TPK1* or *TPK2* leads to overacidification in response to glucose stress. On the other hand, deletion of *TPK3* has the opposite effect, and the functionally important phosphorylation sites do not conform to the PKA consensus sequence (R/K)(R/K)*X*(S/T). The *YCK1* and *YCK2* deletions did not affect pH (Fig. 2).

Whereas the ability of G proteins to control glucose metabolism is well-established, less is known about how changes in glucose metabolism affect G protein structure and function. We and others have shown that pH strongly influences the conformation of G-protein α subunits, both in yeast and in animal cells. pH-dependent changes in Gpa1 and Gα_i were documented by measurements of thermal stability and confirmed by NMR spectroscopy (83, 84). In the case of Gpa1, pH-dependent changes result in increased phosphorylation of the protein and diminished responsiveness to the mating pheromone receptor. Likewise, our computational and biochemical evidence indicates that Gpa2 is a pH sensor. Indeed, our *in vitro* Gpa2 results are consistent with the pH effects associated with Gpa2 overexpression and rescue experiments presented in Fig. 5. These data indicate that there is a correspondence between Gpa2 levels and the degree of intracellular acidification in response to glucose stress. We speculate that the pH-sensing capabilities of Gpa2 establish a positive feedback loop that increases Gpa2 activity as cellular pH drops in response to carbon stress.

Our findings complement those of Dechant *et al.* (76, 88), who showed that glucose limitation leads to the inactivation of another pH regulator, the vacuolar ATP-driven proton pump V-ATPase. Those investigators likewise considered a possible link between pH signaling and the small G proteins Ras1 and Ras2 (76). Whereas deletion of the V-ATPase component Vma2 led to diminished Msn2 translocation and transcriptional induction in response to glucose limitation, there was no change in Ras activation or cAMP abundance (76). Based on those findings and the findings presented here, we conclude that pH acts as a second messenger downstream of the glucose receptor and Ras-mediated signaling pathways. Although a follow-up paper (88) reported a "drastic decrease" in Ras activity in the same *vma2* mutant, those findings relied on a cell-based assay that monitors the location of Ras-binding domain fused to GFP. Interpretation of such experiments is complicated by the fact that GFP fluorescence and the effector-binding region of H-Ras are also sensitive to pH (75, 79, 89).

In conclusion, we propose that pH serves as a "universal" second messenger of glucose availability. Thus, the observed changes in pH could signal the loss of glucose availability and invoke coordinated cellular responses to glucose stress (90–93). Whereas sensors of glucose abundance (Rgt2/Snf3 or Gpr1) are necessary for proton accumulation, the sensor of glu-

cose limitation (Snf1) is needed to prevent overacidification. In contrast to ATP and cAMP, the changes in pH are robust and sustained. Finally, we propose that some sugars regulate cellular acidification in the manner of pharmacological inverse agonists. A challenge for the future is to determine the underlying mechanisms of receptor signaling, the identity of other cellular targets of pH regulation, and their validation as potential targets for human pharmacology.

Experimental procedures

Yeast strains

Strains used in this study were BY4741 (*MAT***a** *leu2*Δ *met15*Δ *his3*Δ *ura3*Δ) and BY4741-derived mutants from the yeast deletion library (Invitrogen). Yeast deletion strains that exhibited pH recovery phenotypes that differed from wildtype by ≥0.2 ΔpH units were remade in our laboratory using the KanMX4 cassette that confers resistance to the antibiotic G418: *hxk2*Δ, *reg1*Δ, *mig1*Δ, *gpr1*Δ, *rgt1*Δ, *rgt2*Δ, *gpa2*Δ, and *snf1*Δ. Additionally, several strains from the yeast deletion library were remade to build a variety of double and triple deletion strains. Remade *gpa2*Δ was used to build the double deletion strain *gpa2*Δ::KanMX4 *gpr1*Δ::*HIS3*. Remade *snf3*Δ was used to build the double deletion strain *rgt2*Δ::*URA3 snf3*Δ::KanMX4 and the triple deletion strain *rgt2*Δ::*URA3 snf3*Δ::KanMX4 *gpr1*Δ::*HIS3*. Remade *tpk1*Δ, *tpk2*Δ, and *tpk3*Δ were used to build the three double deletion strains *tpk1*Δ::KanMX4 *tpk2*Δ::*URA3*, *tpk1*Δ::KanMX4 *tpk3*Δ::*URA3*, and *tpk2*Δ::KanMX4 *tpk3*Δ::*URA3*. Remade *mth1*Δ was used to build the double deletion strain *mth1*Δ::KanMX4 *std1*Δ::*URA3*. Last, the triple deletion strain, *elm1*Δ::*URA3 sac1*Δ::*LEU2 tos3*Δ::KanMX4, was made previously in our laboratory (94).

Plasmids

Plasmids used in this study were pYEplac181 (2μ, amp^R, *LEU2*$^+$) containing the pHluorin gene under control of a constitutive *TEF1* promoter (a gift from Rajini Rao, Johns Hopkins University) and pYEplac195 (2μ, amp^R, *URA3*$^+$) containing genes for *GPA2* or *GPA2*^{Q300L} under control of a constitutive *ADH1* promoter (a gift from Joseph Heitman, Duke University) (95).

Media

All sugar readdition experiments were done in synthetic complete (SC) pH medium: 50 mM dibasic potassium phosphate, 50 mM dibasic sodium succinate, 2% glucose, 1.7 g/liter yeast nitrogen base, 5 g/liter ammonium sulfate, 0.69 g/liter CSM-LEU mixture (complete synthetic medium lacking leucine), titrated to pH 5.0 with HCl and sterile-filtered. For plasmid transformations, strains were grown in YPD (10 g/liter yeast extract, 20 g/liter peptone, 2% glucose), transformed using the LiAC method, and plated on SC dextrose plates (5 g/liter ammonium sulfate, 1.7 g/liter yeast nitrogen base, 1 NaOH pellet, 15 g/liter bacto agar, 2% glucose, and either 0.69 g/liter CSM-LEU for the pHluorin plasmid or 0.67 g/liter CSM-LEU-URA for co-transformation of the pHluorin plasmid and each of the *GPA2* plasmids). CSM mixtures were purchased from MP Biomedicals.

Gpa2 production, purification, and thermal stability measurements

A carboxyl-terminal His_6-tagged version of yeast Gpa2 was subcloned into a pLicHis plasmid for overexpression in *E. coli*. Using methods extensively detailed in our previous studies of G proteins (82, 96), Gpa2 protein was produced via autoinduction, batch nickel-affinity–purified, and thermally unfolded as a function of pH by the fast quantitative cysteine reactivity (fQCR) assay (97).

Measurement of intracellular pH

Intracellular pH was measured using the pH biosensor pHluorin expressed under the control of a constitutive *TEF1* promoter from an episomal plasmid YEplac181 (2μ, amp^R, *LEU2*$^+$). Cells were grown in 2% glucose to $A_{600\,nm}$ ~1.0 at 30 °C, harvested by centrifugation, and washed twice in SC dextrose pH medium lacking glucose. In a 96-well microplate, 180 μl of washed cells were combined with 20 μl of 10% (w/v) glucose using a multichannel pipette to achieve final glucose concentrations of 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, and 0% (w/v). The samples were then mixed five times using a multichannel pipette and immediately placed in a SpectraMax fluorescence plate reader to measure pH recovery kinetics over 30 min at an ambient reader temperature of 22 °C. This process had a dead time of 30 s. As described previously in detail (82), the pHluorin biosensor provides a ratiometric readout of pH; using a standard curve, intracellular pH values are calculated from the emission ratio at 520 nm (r) in response to excitation at 395 and 480 nm (*i.e.* $r = 395^{@520\,nm}/480^{@520\,nm}$). In our hands, pHluorin-based pH measurements are highly reproducible, with experimental uncertainties usually <0.02 pH units.

Sugar-mixing experiments

Cells containing pHluorin were grown in 2% glucose to an $A_{600\,nm}$ of 1.0 at 30 °C. Cells were harvested and washed by transferring 1.0 ml of culture to several 1.5-ml tubes, centrifuging for 30 s at 21,000 relative centrifugal force, aspirating the supernatant, and resuspending the cell pellet in 1.0 ml of low fluorescence SC pH medium lacking glucose and made with low fluorescence yeast nitrogen base lacking folic acid and riboflavin (Formedium CYN6505). This process was repeated once more to remove any residual glucose. A pairwise sugar combination matrix of fructose, galactose, glucose, maltose, mannose, and sucrose was mixed in a 96-well microplate (Greiner 96 F-bottom; 655209) by combining 160 μl of washed cells with 20 μl of a 16% (w/v) solution of each pairwise sugar (final sugar concentrations of 1.6%). For example, the fructose and glucose pairwise mixture contained 160 μl of cells in low fluorescence SC pH medium, 20 μl of a 16% (w/v) fructose, and 20 μl of 16% (w/v) glucose. Medium blanks for background subtraction were composed of 160 μl of low fluorescence SC pH medium combined with 40 μl of water. The mixed sugar matrix was incubated for 10 min at room temperature, and pHluorin fluorescence was quantified using a ClarioStar plate reader (excitation wavelengths of 385 and 475 nm, and bandpass filter of 15 nm; dichroic filter of 500 nm; emission wavelength of 528 nm and bandpass filter of 16 nm; matching channel gains of 1,800; 40 flashes/well; and orbital averaging with a scan diameter of 3

mm). All pHluorin measurements were made within 10 min of sugar readdition to avoid the onset of invertase enzymatic activity (85).

Structural informatics

PDB (98) identifiers for the 112 Gα structures analyzed in this study were obtained from the SMART database (http://smart.embl-heidelberg.de)[4] using the domain identifier *G_alpha* (99, 100). Using the Protein Data Bank's advanced search feature, these PDB identifiers were batch-queried to build a custom table of CATH (http://www.cathdb.info)[4] (101, 102) and PFAM (http://xfam.org)[4] (103) identifiers (Table S2). This table was used to organize each Gα subunit by PDB identifier, protein chain, and domain (Table S3). pHinder and consensus network analysis calculations were performed exactly as described previously (96). The yeast Gpa2 homology model was built using Swiss-Model (http://swissmodel.expasy.org)[4] by threading the Gpa2 primary sequence, obtained from the Saccharomyces Genome Database (http://www.yeastgenome.org),[4] onto the template structure corresponding to chain A of the PDB identifier 4N0D (*Rattus norvegicus* $G\alpha_{i1}$ subunit) (104).

Sample preparation for measuring intracellular ATP concentration

Cells were grown to a $A_{600\,nm}$ of ~1.0, collected by centrifugation, and resuspended twice in SC medium and a third time in SC medium plus either 1.6% or 0.05% glucose. After 10 min, the cells were again collected, snap-frozen, and then resuspended in 200 μl of water containing stable isotope-labeled ATP and AMP and boiled for 10 min. Samples of growth medium were collected in parallel to confirm changes in extracellular glucose and to establish background levels of adenosine metabolites. Cell lysates were centrifuged at 14,800 $\times$ *g* at 4 °C, and the supernatant was removed for LC-MS analysis. LC-MS samples contained 25% (v/v) supernatant and 75% acetonitrile (v/v).

HPLC-MS/MS quantification of intracellular glucose, ATP, and cAMP

Mass spectrometry experiments were performed on a Thermo Fisher TSQ-Quantum Ultra triple-quadrupole mass spectrometer coupled to a Surveyor HPLC system following the method of Johnsen *et al.* (105). Chromatography was performed on a Sequant ZIC-pHILIC 2.1 $\times$ 150-mm, 5-μm column (MilliporeSigma, Billerica, MA) held at 15 °C using a mobile phase of 100 mM ammonium bicarbonate (Sigma-Aldrich) in deionized water (generated on-site using a filtration system from Pure Water Solutions, Hillsborough, NC) and acetonitrile (Fisher Optima™, Thermo Fisher Scientific, Waltham, MA) flowing at 0.2 ml/min. The aqueous portion was held at 25% from 0 to 2 min, increased linearly to 50% from 2 to 8 min, held at 50% from 8 to 12 min, decreased to 25% from 12 to 12.5 min, and then held at 25% until 15 min. Injection volume was 10 μl, and column effluent was diverted to waste for the first 2.3 min of each run. Analytes and stable isotope-labeled internal standards were detected in negative mode using mass transitions and collision energies as listed in Table S1. Electrospray conditions were as follows: spray voltage of 3000 V, vaporizer (HESI) temperature of 200 °C, sheath gas pressure 40, auxiliary gas pressure 20, ion sweep gas pressure 1.0, capillary temperature of 350 °C, and argon collision gas pressure of 1.5 millitorrs.

Reported peak areas for each sample set were generated by calculating the peak area ratio of analyte to internal standard. Calculations for ATP used $^{13}C_{10}{}^{15}N_5$-ATP, and calculations for cAMP and glucose used $^{13}C_{10}{}^{15}N_5$-AMP as an internal standard. Xcaliber version 3.0.63 was used for acquisition and data analysis. Internal standards (ATP and AMP) and analyte standards (cAMP, ATP, and glucose) were purchased from Sigma-Aldrich.

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