# Phosphorylation of the Gα protein Gpa2 promotes protein kinase A signaling in yeast

Received for publication, May 30, 2019, and in revised form, October 31, 2019 Published, Papers in Press, November 5, 2019, DOI 10.1074/jbc.RA119.009609

**Shan Huang, Alex Benben, Robert Green, Nina Cheranda, Grace Lee, Benita Joseph, Shannon Keaveney, and Yuqi Wang**[1]

*From the Department of Biology, Saint Louis University, St. Louis, Missouri 63103*

Edited by Henrik G. Dohlman

**Heterotrimeric G proteins are important molecular switches that facilitate transmission of a variety of signals from the outside to the inside of cells. G proteins are highly conserved, enabling study of their regulatory mechanisms in model organisms such as the budding yeast *Saccharomyces cerevisiae*. Gpa2 is a yeast Gα protein that functions in the nutrient signaling pathway. Using Phos-tag, a highly specific phosphate binding tag for separating phosphorylated proteins, we found that Gpa2 undergoes phosphorylation and that its level of phosphorylation is markedly increased upon nitrogen starvation. We also observed that phosphorylation of Gpa2 depends on glycogen synthase kinase (GSK). Disrupting GSK activity diminishes Gpa2 phosphorylation levels *in vivo*, and the purified GSK isoforms Mck1 and Ygk3 are capable of phosphorylating Gpa2 *in vitro*. Functionally, phosphorylation enhanced plasma membrane localization of Gpa2 and promoted nitrogen starvation–induced activation of protein kinase A. Together, the findings of our study reveal a mechanism by which GSK- and nutrient-dependent phosphorylation regulates subcellular localization of Gpa2 and its ability to activate downstream signaling.**

Heterotrimeric G proteins are highly conserved molecular switches that regulate a variety of cellular processes (1–3). The strength and duration of G protein signaling have to be tightly regulated, and their alteration can have significant impact on pathophysiology and pharmacology (4–6). Our goal is to elucidate molecular mechanisms that regulate G proteins, with a focus on the role of posttranslational modifications, especially those that are responsive to environmental signals.

Gpa2 is a Gα protein in the budding yeast *Saccharomyces cerevisiae*, and it primarily functions in the nutrient signaling pathway (7, 8). In the presence of glucose, Gpr1, a G protein–coupled receptor, becomes activated, which, in turn, facilitates guanine nucleotide exchange of Gpa2 from the GDP-bound form to the GTP-bound form (8, 9). GTP-bound Gpa2 then activates the adenylate cyclase Cyr1 to increase production of cAMP (7, 10), an important second messenger that activates PKA to regulate cell growth and proliferation. Given the pivotal role of Gpa2 in the nutrient signaling pathway, its activity has to be tightly regulated. One well-established mechanism for Gpa2 regulation is action of the regulator of G protein signaling (RGS) protein Rgs2 (11), which accelerates the GTPase activity of Gpa2 and keeps its signaling in check. Other mechanisms for Gpa2 regulation likely exist. An earlier study implied that Gpa2 may be phosphorylated when diploid cells undergo nitrogen deprivation–induced sporulation and meiosis (12). More recently, global phosphoproteomics studies also revealed Gpa2 to be a potential phosphoprotein (13). Thus, it appears that Gpa2 and Gpa2-mediated signaling may be regulated via phosphorylation.

In this study, we found that Gpa2 protein indeed undergoes phosphorylation. Interestingly, nitrogen starvation markedly elevates the level of Gpa2 phosphorylation and enhances its plasma membrane localization. We also found that preventing Gpa2 phosphorylation by mutating its candidate phosphorylation sites diminishes the level of nitrogen starvation–induced activation of protein kinase A. Our results suggest that nitrogen starvation–induced phosphorylation of Gpa2 regulates subcellular localization of Gpa2 and enhances its ability to activate downstream protein kinase A signaling.

## Results

### *Gpa2 undergoes phosphorylation*

To examine whether Gpa2 is phosphorylated, we made use of a yeast strain that expresses a GFP tag at the C-terminal end of Gpa2 at its genomic locus (14). Cell extracts from Gpa2-GFP cells treated or not treated with nitrogen starvation were analyzed on a regular gel as well as a gel containing Phos-tag. The nitrogen starvation condition was chosen because an earlier work implied that that Gpa2 may undergo phosphorylation under sporulation-inducing conditions (12). Phos-tag is a small chemical compound that forms a specific binding site for a phosphate group in the presence of divalent metal ions such as $Zn^{2+}$; thus, SDS-PAGE using an acrylamide gel that contains Phos-tag can specifically retard the migration of phosphorylated protein species (15, 16). As shown in Fig. 1*A*, on a regular gel, Gpa2-GFP ran as a single band. Interestingly, on a Phos-tag gel, extra and slower-migrating bands were visible for Gpa2-GFP, especially in samples prepared from cells that were subject to nitrogen starvation.

Because the C terminus of Gα protein is critical for its function, which includes coupling to receptors (17–19), we wished

This work was supported by NIGMS, National Institutes of Health Grant R15GM128047-01 (to Y. W.) and Knoedler undergraduate research funds from Saint Louis University (to A. B., S. K., and B. J.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[1] To whom correspondence should be addressed: Dept. of Biology, Saint Louis University, 3507 Laclede Ave., St. Louis, MO 63103. Tel.: 314-977-4178; Fax: 314-977-3658; E-mail: yuqi.wang@slu.edu.

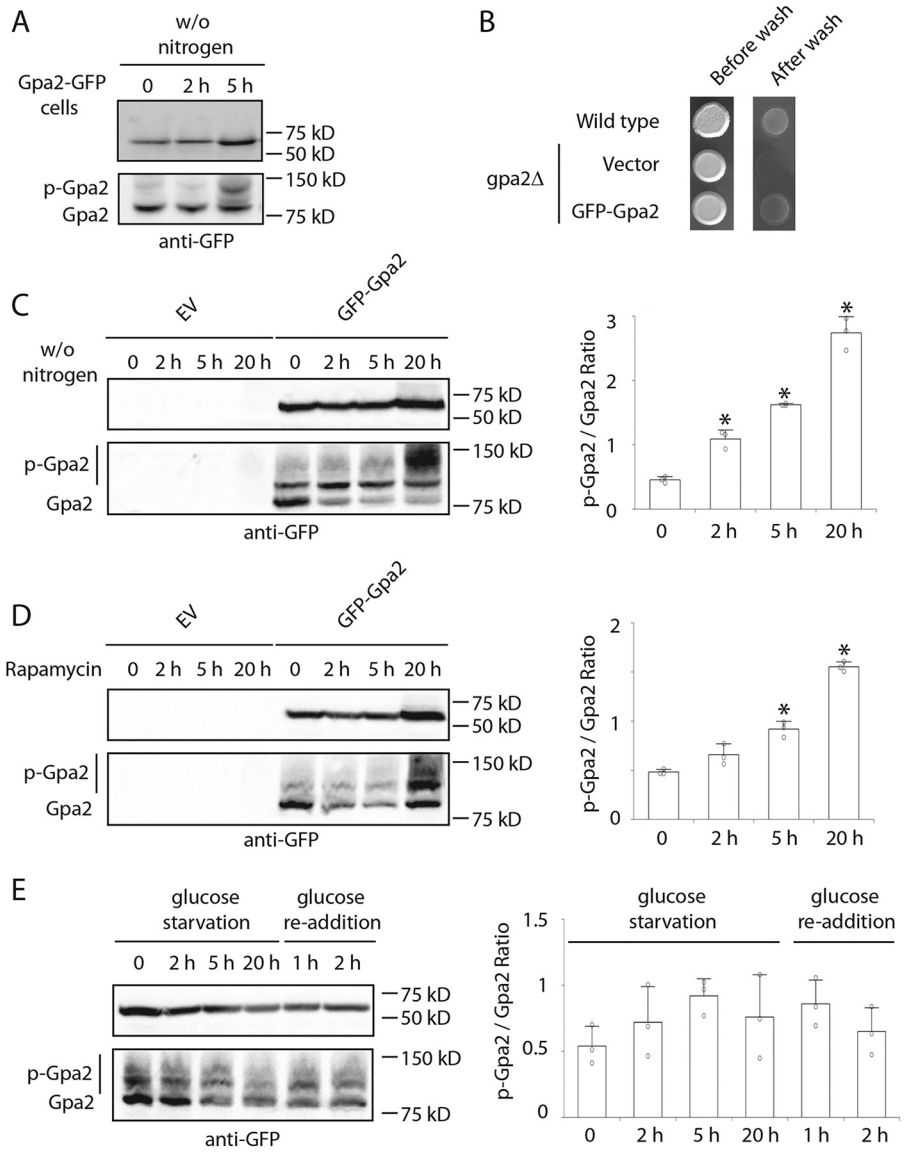


**Figure 1. Phosphorylation of Gpa2 and its regulation by nutrient status.** *A*, Gpa2-GFP cells with GFP tagged at the C terminus of Gpa2 at its genomic locus were grown to mid-log phase and shifted to nitrogen starvation conditions for the indicated times. Whole-cell extracts were resolved on 8% regular (*top*) or Phos-tag– containing (*bottom*) SDS-PAGE and probed with anti-GFP antibodies (ab13970, Abcam). *p-Gpa2*, phosphorylated Gpa2. *B*, the Σ1278-based invasive strain lacking *GPA2* was transformed with either empty vector or plasmids that express $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ under control of the *GPA2* promoter. Overnight cultures were spotted onto a yeast extract/peptone/dextrose plate, grown at 30 °C for 2 days, and gently washed with water to reveal the invasive growth. The plates were photographed before and after washing. *C*, *gpa2*Δ cells transformed with either empty vector (EV) or plasmids expressing $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ were grown to mid-log phase and shifted to nitrogen starvation conditions for the indicated times. Whole-cell extracts were resolved on 8% regular (*top*) or Phos-tag– containing (*bottom*) SDS-PAGE and probed with anti-GFP antibodies. Quantification of immunoblots by densitometry from three independent experiments is shown in the *right panel*. The difference between each time point and time 0 was statistically analyzed (*, $p < 0.050$). *D*, the same cells as in *C* were grown to mid-log phase and treated with rapamycin (0.2 μg/ml) for 2 h. Whole-cell extracts were resolved on either regular or Phos-tag–containing SDS-PAGE and probed with anti-GFP antibodies. Quantification of immunoblots by densitometry from three independent experiments is shown in the *right panel*. The difference between each time point and time 0 was statistically analyzed (*, $p < 0.050$). *E*, *gpa2*Δ cells transformed with plasmids expressing $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ were grown to mid-log phase, switched to medium without glucose (*glucose starvation*) for the indicated time, and then shifted to medium with glucose (*glucose re-addition*). Whole-cell extracts were resolved on either regular or Phos-tag– containing SDS-PAGE and probed with anti-GFP antibodies. Quantification of immunoblots by densitometry from three independent experiments is shown in the *right panel*.

to examine whether the position of the GFP tag matters for the observed phosphorylation. For this purpose, we made a construct that expresses N-terminally tagged Gpa2. Because the first few residues in the N-terminal region of Gpa2 are important for its myristoylation and membrane localization (20–22), the GFP tag was inserted after the 10th residue of the Gpa2 protein, as described earlier (20). The resulting $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ protein has been demonstrated previously to be fully functional and can replace WT Gpa2 (20). To verify this, we examined the ability of $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ to support invasive growth, a phenomenon that occurs in haploid yeast under nutrient depletion conditions and requires functional Gpa2 (23). As shown in Fig. 1*B*, disrupting Gpa2 severely impaired invasive growth, as expected, and expressing $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ restored the ability of the *gpa2*Δ cells to promote invasive growth, indicating that the fusion protein is functional. We then examined phosphorylation of this N-terminally GFP-tagged Gpa2. As shown in Fig. 1*C*, phosphoryla-

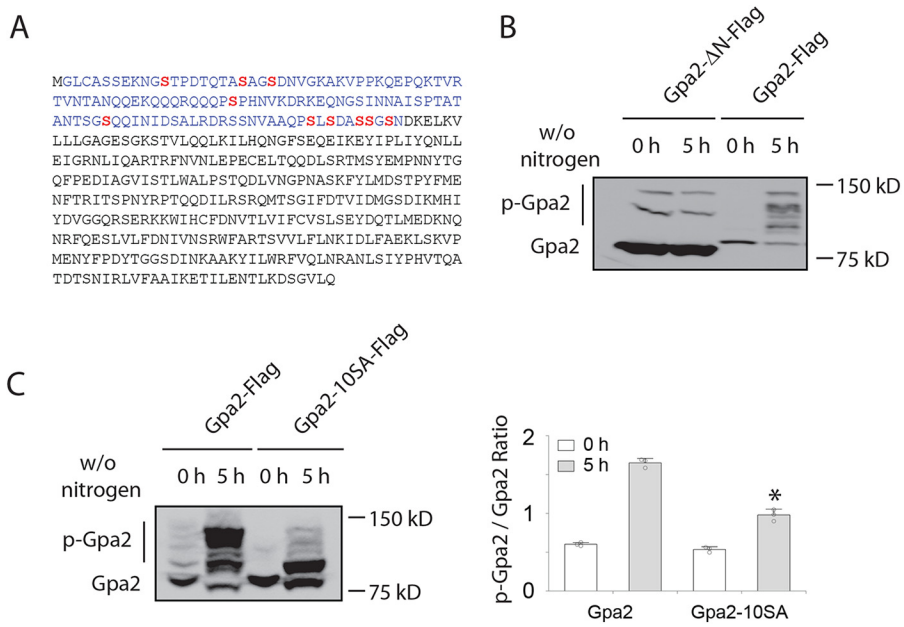


**Figure 2. Phosphorylation of Gpa2 requires its N-terminal region.** *A*, the amino acid sequence of Gpa2, with phosphorylation sites revealed by phosphoproteomics studies highlighted in *red*. The region highlighted in *blue* was removed when generating the Gpa2-ΔN-FLAG construct. *B*, whole-cell extracts from cells expressing either full-length Gpa2 (*Gpa2-FLAG*) or a Gpa2 variant lacking the first 120 amino acid residues (*Gpa2-ΔN-FLAG*) were separated on Phos-tag–containing SDS-PAGE and immunoblotted to reveal Gpa2. The data shown are representative of three independent experiments. *C*, whole-cell extracts from cells expressing either WT Gpa2 or a Gpa2–10SA mutant were separated on Phos-tag– containing SDS-PAGE and immunoblotted to reveal Gpa2. Quantification of immunoblots by densitometry from three independent experiments is shown in the *right panel*. The difference between Gpa2 and Gpa2–10SA at time 5 h was statistically analyzed (*, $p < 0.050$).

tion of $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ was readily detectable, and again, the level of phosphorylation was markedly elevated upon nitrogen starvation. Given that nitrogen starvation conditions can be mimicked by rapamycin treatment, we also examined whether rapamycin treatment can promote Gpa2 phosphorylation. As shown in Fig. 1*D*, rapamycin treatment can indeed stimulate Gpa2 phosphorylation, but to a somewhat lesser extent than nitrogen starvation.

Next we sought to examine whether phosphorylation of Gpa2 is sensitive to the availability of other nutrients, such as glucose. For this purpose, cells expressing $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ protein were grown to mid-log phase, shifted to medium without glucose for the indicated time (glucose starvation), and then shifted back to medium with glucose (glucose re-addition). As shown in Fig. 1*E*, altering the level of glucose (either removal or re-addition) had very little effect on the extent of Gpa2 phosphorylation. Together, these results indicate that Gpa2 protein undergoes phosphorylation and that its phosphorylation is sensitive to the availability of nitrogen.

### *Phosphorylation-null mutant of Gpa2*

To start characterizing Gpa2 phosphorylation, we sought to identify the regions on Gpa2 that are required for its phosphorylation. Global phosphoproteomics studies have indicated that several residues in the N-terminal region of Gpa2 may undergo phosphorylation (Fig. 2*A*) (13). Thus, we first examined whether the N-terminal region of Gpa2 is critical for its phosphorylation. To this end, we generated a truncated version of Gpa2 that lacks a stretch of 120 residues from the N-terminal region of the protein. As shown in Fig. 2*B*, nitrogen starvation–induced phosphorylation was absent in this N-terminally truncated version of Gpa2, suggesting that sites of Gpa2 phosphorylation may indeed reside in the N-terminal region of the protein. To further test this, we constructed the Gpa2–10SA mutant, in which a total of 10 serine residues (Ser-12, Ser-20, Ser-23, Ser-61, Ser-90, Ser-111, Ser-113, Ser-116, Ser-117, and Ser-119) present in the N-terminal region of the protein were replaced with alanine. Those residues were chosen because they either represent phosphorylation sites revealed by phosphoproteomics studies (13) or are adjacent to those sites. As shown in Fig. 2*C*, the Gpa2–10SA mutant also displayed a much diminished level of phosphorylation induced by nitrogen starvation, especially the species that run very slowly and presumably represent multiply phosphorylated species.

### *Glycogen synthase kinases are required for Gpa2 phosphorylation*

Next we sought to identify the kinase that is responsible for Gpa2 phosphorylation. One candidate is glycogen synthase kinase (GSK),[2] because GSK is known to be active under nitrogen starvation conditions, and there are predicted GSK phosphorylation sites on Gpa2 (24, 25). There are a total of four GSK isoforms in yeast: Mrk1, Mck1, Rim11, and Ygk3 (YOL128C). These GSK isoforms have partially redundant functions in phosphorylating substrates (24, 26). To determine whether GSK activity is required for Gpa2 phosphorylation, we made use of a strain that lacks three GSK isoforms (Mck1, Rim11, and Ygk3) and has been used as a gsk-null strain (26). As shown in Fig. 3*A*,

[2] The abbreviations used are: GSK, glycogen synthase kinase; MAPK, mitogen-activated protein kinase; EV, empty vector; CFP, cyan fluorescent protein; RFP, red fluorescent protein.

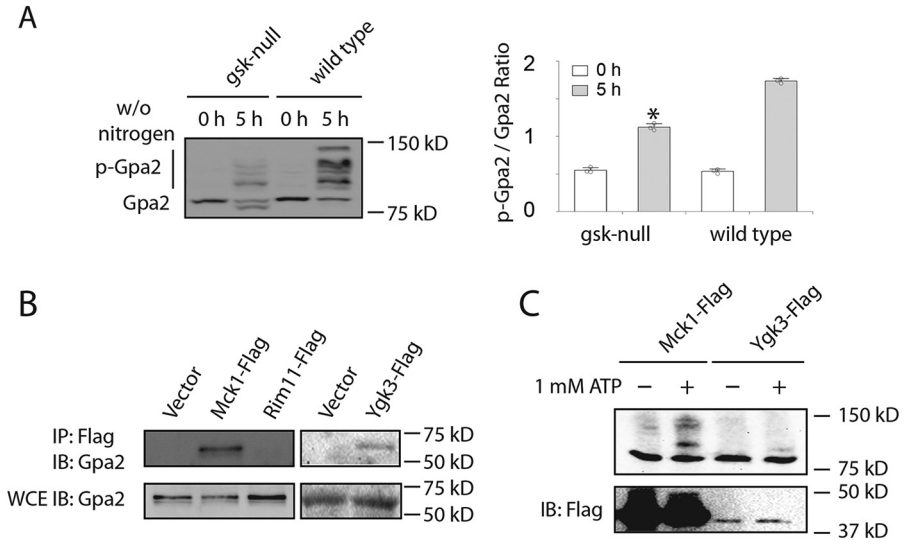


**Figure 3. Phosphorylation of Gpa2 is GSK-dependent.** *A*, cells lacking three GSK isoforms (Mck1, Rim11, and Ygk3) and its isogenic WT were grown to mid-log phase and treated or not treated with nitrogen starvation. Whole-cell extracts were separated on Phos-tag–containing SDS-PAGE gel, and Gpa2 was detected via immunoblotting. Quantification of immunoblots by densitometry from three independent experiments is shown in the *right panel*. The difference between gsk-null and WT at time 5 h was statistically analyzed (*, $p < 0.050$). *B*, interaction between FLAG-tagged Mck1, Rim11, or Ygk3 with GFP-tagged Gpa2 was analyzed by immunoprecipitation (*IP*) of FLAG-tagged protein and immunoblotting (*IB*) of GFP-tagged Gpa2. The data shown are representative of three independent experiments. *WCE*, whole-cell extract. *C*, FLAG-tagged Mck1 and Ygk3 were immunopurified from cells that were nitrogen-starved for 12 h. The purified proteins were mixed with immunopurified GFP-tagged Gpa2 in the presence or absence of ATP, and the resulting phosphorylated Gpa2 was monitored using Phos-tag SDS-PAGE. The data shown are representative of three independent experiments.

the level of Gpa2 phosphorylation is substantially diminished in the gsk-null strain, indicating that GSK activity is required for full phosphorylation of Gpa2. To determine whether the requirement of GSK on Gpa2 phosphorylation is direct, we first examined whether Gpa2 interacts with any of the GSK isoforms. For this purpose, we made a FLAG-tagged versions of Mck1, Rim11, and Ygk3 and examined the interaction between Gpa2 and Mck1/Rim11/Ygk3 using coimmunoprecipitation. As shown in Fig. 3*B*, we were able to detect interaction between Mck1 and Gpa2 as well as Ygk3 and Gpa2. Next we tested whether Mck1 and Ygk3 are capable of phosphorylating Gpa2. For this purpose, we purified Mck1-FLAG and Ygk3-FLAG from cells under nitrogen starvation conditions (to maximize its kinase activity) and GFP-tagged Gpa2 from normally growing cells and conducted an *in vitro* kinase assay. As shown in Fig. 3*C*, in the presence of both ATP and Mck1-FLAG, an increase in phosphorylated Gpa2 was observed. Likewise, the presence of both ATP and Ygk3-FLAG also led to a subtle increase in the level of Gpa2 phosphorylation. For reasons that are not clear, the expression level of Ygk3 was much lower than that of Mck1, which may explain the lesser extent of Gpa2 phosphorylation caused by Ygk3. From these data, we conclude that Mck1 and Ygk3 are capable of phosphorylating Gpa2. Together, these findings support that glycogen synthase kinases are responsible for Gpa2 phosphorylation.

### *Subcellular localization of Gpa2*

Accumulating evidence indicates that G proteins can function in both the plasma membrane and other subcellular compartments, including endosomes and the Golgi (27, 28). In principle, altering subcellular localization of G proteins could either enable or impair their ability to regulate specific effectors by either facilitating G protein/effector interaction or sequestering the G protein away from its effector. To investigate whether Gpa2 may be regulated via this mechanism, we examined the effect of nitrogen starvation as well as phosphorylation on subcellular localization of GFP-tagged Gpa2, *i.e.* $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$. As shown in Fig. 4*A*, in cells grown in normal medium, a fluorescence signal representing GFP-tagged Gpa2 can be found in the plasma membrane and cytoplasm. Upon nitrogen starvation, a clear concentration of Gpa2 signal on the plasma membrane was observed, indicating that nitrogen starvation enhances plasma membrane localization of Gpa2. In addition, clusters of fluorescent signals are present in starved cells that presumably represent free GFP or remnants of GFP-Gpa2 in the vacuole as a consequence of nitrogen starvation–induced autophagy. Interestingly, nitrogen starvation has less of an effect on localization of the Gpa2–10SA mutant, suggesting that nitrogen starvation–induced alteration of Gpa2 localization is phosphorylation-dependent. To confirm the effect of nitrogen starvation and phosphorylation on Gpa2 subcellular localization, we also subjected cells expressing $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ to sucrose density gradient fractionation analysis (29). Consistent with our fluorescence imaging results, a substantial fraction of GFP-tagged Gpa2 was present in the cytosolic fraction and the plasma membrane fractions (Fig. 4*B*). Nitrogen starvation clearly induced a shift of GFP-tagged Gpa2 from cytosolic fractions to plasma membrane fractions, and this effect was not apparent in the GFP-tagged Gpa2–10SA mutant (Fig. 4*B*).

To determine whether nitrogen starvation induces general enrichment of protein to the plasma membrane, we also examined the subcellular localization of Gpa1, the only other G$\alpha$ protein in yeast. A yeast strain expressing CFP-tagged Gpa1 was used for this purpose. This strain was created for FRET analysis

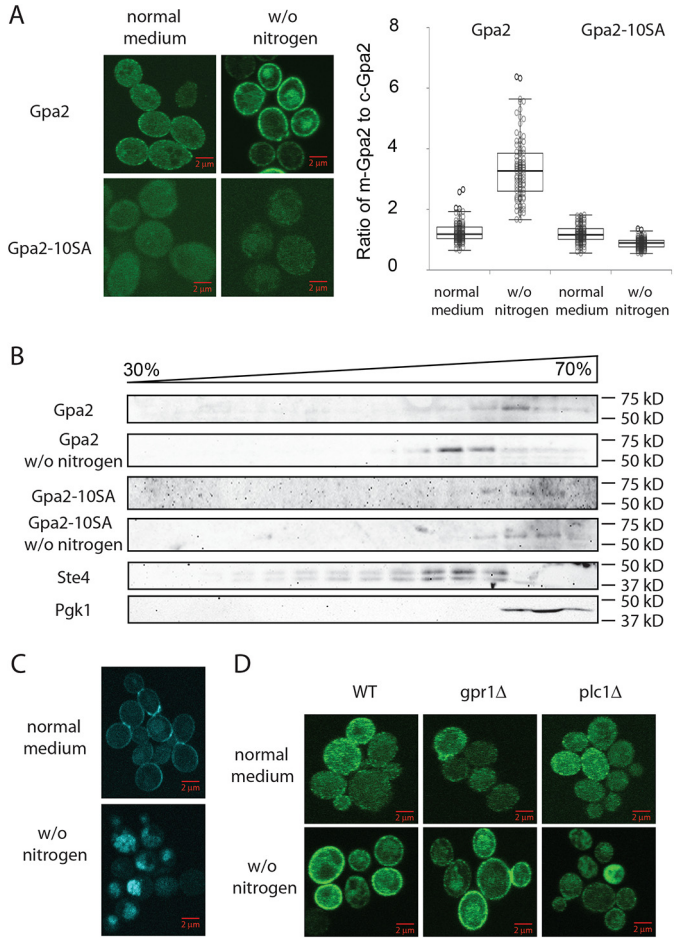


**Figure 4. Subcellular localization of Gpa2.** *A*, *gpa2*Δ mutant cells expressing either Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$ or Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$–10SA were grown in normal medium to mid-log phase, and the localization of GFP-tagged Gpa2 was visualized using confocal microscopy. A portion of mid-log-phase cells was switched to nitrogen starvation medium, incubated for 6 h, and imaged similarly. Quantification of immunofluorescence signals was conducted using ImageJ. A small section of plasma membrane and cytoplasm from each cell was selected for density measurements, and their ratio was calculated. Data from 100 cells are shown in the *right panel*. *m-Gpa2*, density of plasma membrane–localized Gpa2; *c-Gpa2*, density of cytoplasmic localized Gpa2. *B*, cells expressing either Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$ or Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$–10SA were grown to mid-log phase and then subjected to nitrogen starvation treatment for 6 h. Whole-cell extracts were prepared, separated by sucrose gradient fractionation, resolved by 8% SDS-PAGE, and probed with anti-GFP, anti-Ste4, or anti-Pgk1 antibodies. *C*, cells expressing CFP-Gpa1 were grown in normal medium to mid-log phase, and the localization of CFP-tagged Gpa1 was visualized using confocal microscopy. *D*, WT or mutants lacking *GPR1* or *PLC1* were transformed with plasmids expressing Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$ were imaged similarly as described in *A*. The data shown are representative of three independent experiments.

of G protein activation (30), and, presumably, the CFP-tagged Gpa1 is fully functional. As shown in Fig. 4*C*, in cells grown in normal medium, CFP-Gpa1 was primarily on the plasma membrane, with some cytoplasmic localization; when treated with nitrogen starvation, the CFP-Gpa1 signal was clustered in the cells, and no enrichment on plasma membrane was observed, which is very different from what was found in Gpa2.

It is possible that the apparent enrichment of Gpa2 from the cytoplasm to the plasma membrane in response to nitrogen starvation requires the presence of its interacting proteins in the plasma membrane. Gpr1 and Plc1 are two plasma membrane–associated proteins that are known to interact with Gpa2 (31, 32). Gpr1 acts as a receptor exchange factor for Gpa2, whereas Plc1 interacts with both Gpr1 and Gpa2 and serves to enhance the interaction between Gpr1 and Gpa2 by exposing a Gpa2 binding site at the C-terminal region of Gpr1 (32). To determine whether these two proteins are required for Gpa2 localization to the plasma membrane, we examined the effect of disrupting their genes. As shown in Fig. 4*D*, disrupting Gpr1 has no effect on subcellular localization of Gpa2. However, in the *plc1*Δ mutants, nitrogen starvation–induced plasma membrane enrichment of Gpa2 is much less pronounced, suggesting a potential role of Plc1 in anchoring Gpa2 on the plasma membrane upon nitrogen starvation.

### *Functional consequences of Gpa2 phosphorylation*

Finally, we wished to investigate the impact of phosphorylation on Gpa2-mediated signaling outputs. To this end, we first examined whether WT Gpa2 and the Gpa2–10SA mutant show any difference in their behavior in promoting invasive growth. As shown in Fig. 5*A*, disrupting Gpa2 severely impaired invasive growth, as expected. Compared with cells expressing WT Gpa2, cells that expressed the Gpa2–10SA mutant displayed a similar level of invasive growth, suggesting that phosphorylation is not important to promote haploid invasive growth. This result also indicates that the Gpa2–10SA protein is functional, as it is clearly able to fully support the invasive growth.

Next we examined whether phosphorylation of Gpa2 has any effect on the level of PKA activation, as one important downstream signaling component of Gpa2 is protein kinase A (33). For this purpose, we utilized Myc-Cki, a well-established PKA activity reporter whose level of phosphorylation is directly proportional to the activity of PKA (34). As shown in Fig. 5*B*, basal PKA activity is very similar for Gpa2 and Gpa2–10SA. As reported previously, nitrogen starvation induces a clear elevation of PKA activity (34), and this effect is more prominent in cells expressing Gpa2 than those expressing Gpa2–10SA, suggesting that phosphorylation is required for full activation of PKA induced by nitrogen starvation (Fig. 5*B*). No difference was observed between Gpa2 and Gpa2–10SA with regarding to the level of MAPK activation, as measured by the extent of phospho-Mpk1 and phospho-Kss1 (Fig. 5*B*). This was expected, as MAPKs are not downstream components of the Gpa2 pathway like protein kinase A (35, 36).

Last, we examined whether phosphorylation of Gpa2 has any impact on sporulation. This is possible because Gpa2 is known to interact with Ime2, a kinase that promotes sporulation of diploid yeast in response to nitrogen starvation and the presence of nonfermentable carbon such as acetate (12). It has been suggested that, under nutrient-rich conditions, Gpa2 is capable of inhibiting Ime2 and preventing inappropriate sporulation (12). Presumably, this inhibitory effect of Gpa2 has to be relieved when cells undergo sporulation. Phosphorylation of Gpa2 could potentially serve as a mechanism to relieve the inhibitory effect of Gpa2 on cytoplasmic Ime2 by enhancing the plasma membrane localization of Gpa2. To test this, we first examined whether nitrogen starvation also induces phosphorylation and enhances plasma membrane localization of Gpa2 in diploid cells. Diploid cells expressing Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$ were grown into mid-log phase and subjected to nitrogen starva-

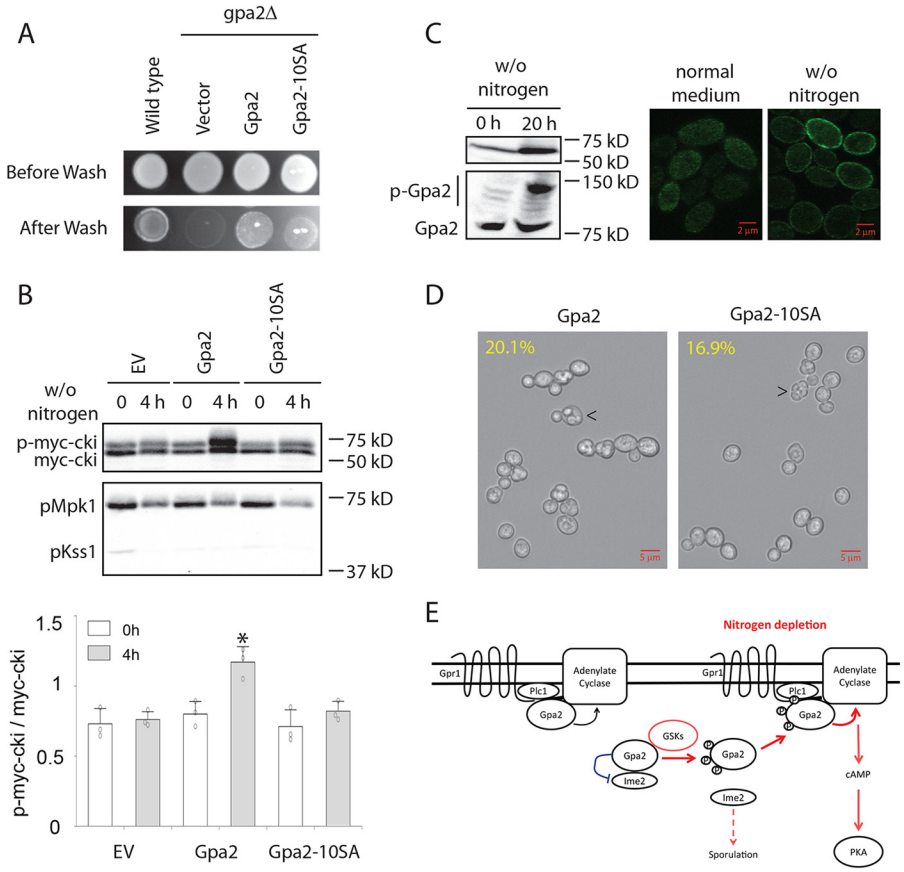


**Figure 5. Functional consequences of Gpa2 phosphorylation.** *A*, the Σ1278-based invasive strain lacking *GPA2* was transformed with either empty vector or plasmids that expressed WT Gpa2 or Gpa2–10SA under control of the *GPA2* promoter. Overnight cultures were spotted onto YPD plates, grown at 30 °C for 2 days, and gently washed with water to reveal the invasive growth. The plates were photographed before and after washing. The data shown are representative of three independent experiments. *B*, BY4741-derived *gpa2*Δ mutant cells expressing either EV, WT Gpa2, or Gpa2–10SA under control of the *GPA2* promoter were grown to mid-log phase and treated or not treated with nitrogen starvation. Whole-cell extracts were separated by 8% SDS-PAGE and probed for myc-Cki and phospho-MAPKs. Quantification of immunoblots by densitometry from three independent experiments is shown in the *bottom panel*. The difference between Gpa2 at time 0 h and 4 h was statistically analyzed (*, $p < 0.050$). *C*, BY4743-derived *gpa2*Δ mutant cells (diploid) expressing $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ were grown to mid-log phase and subjected to nitrogen starvation for 12 h. Phosphorylation of $Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ as well as localization of the protein were analyzed. The data shown are representative of three independent experiments. *D*, BY4743-derived *gpa2*Δ mutant cells expressing either EV, WT Gpa2, or Gpa2–10SA under control of the *GPA2* promoter were subjected to a sporulation assay. Cells with clear signs of sporulation (*arrowheads*) were counted. The difference between Gpa2 and Gpa2–10SA was statistically analyzed ($p = 0.10$). *E*, model of Gpa2 regulation by phosphorylation. Gpa2 is present in both the cytoplasm and the plasma membrane. In the cytoplasm, Gpa2 binds and inhibits Ime2, a kinase critical for sporulation. On the plasma membrane, Gpa2 binds and activates adenylate cyclase, leading to PKA activation. Upon nitrogen starvation, activated GSKs phosphorylate Gpa2 and enhance plasma membrane localization of Gpa2. As a result, nitrogen starvation–induced phosphorylation of Gpa2 diminishes its inhibition of the cytoplasmic effector Ime2 and increases its activation of plasma membrane–localized effector adenylate cyclase, promoting sporulation and PKA activation. The *red arrows* indicate steps that are enhanced by nitrogen starvation.

tion. As shown in Fig. 5*C*, nitrogen starvation similarly induced Gpa2 phosphorylation and plasma membrane localization. We then examined the sporulation efficiency of diploid cells that expressed either WT Gpa2 or the Gpa2–10SA mutant. Cells with a clear sign of the presence of spores (Fig. 5*D*) were counted under a microscope. We found that about 20.1% of cells (of 1005 counted cells) expressing WT Gpa2 underwent sporulation, and about 16.9% of cells (of 706 counted cells) expressing the Gpa2–10SA mutant underwent sporulation. Thus, diminishing the level of Gpa2 phosphorylation had a modest inhibitory effect on sporulation efficiency.

## Discussion

Gpa2 is a Gα protein that regulates nutrient signaling in yeast. In this work, we report that Gpa2 protein undergoes nutrient-regulated phosphorylation. Specifically, nitrogen starvation induces Gpa2 phosphorylation and enhances its localization on the plasma membrane. The Gpa2 mutant with decreased phosphorylation shows a reduced level of PKA activation induced by nitrogen starvation and lower sporulation efficiency. Our results suggest phosphorylation as a mechanism to regulate subcellular localization and signaling of Gpa2.

One interesting observation from our study is that nitrogen starvation induces enhanced plasma membrane localization of Gpa2 (Fig. 4). The difference in Gpa2 localization with and without nitrogen starvation is very clear. A substantial portion of Gpa2 is present in the cytoplasm in cells grown in normal medium, but in nitrogen-starved cells, there exists a substantially lower level of cytoplasmic Gpa2 and a higher level of plasma membrane Gpa2. Thus, nitrogen starvation clearly induces translocation of Gpa2 from the cytoplasm to the plasma membrane. Notably, this effect is fairly specific to Gpa2, as nitrogen starvation does not increase plasma membrane localization of Gpa1, the other Gα protein in yeast. As Gpa2 has

effector proteins both on the plasma membrane (*i.e.* the adenylate cyclase Cyr1) and in the cytoplasm (*i.e.* Ime2), such an alteration in Gpa2 localization can, in principle, impact the interactions between Gpa2 and its effectors, which, in turn, modulates Gpa2-mediated signaling (Fig. 5*E*). Specifically, enhanced plasma membrane localization will facilitate the interaction between Gpa2 and the adenylate cyclase Cyr1, which could lead to increased activity of the downstream protein kinase A. Likewise, diminished cytoplasmic localization of Gpa2 reduces the interaction between Gpa2 to Ime2, a cytoplasmic localized protein kinase important for sporulation (37, 38), relieving the inhibitory effect of Gpa2 on Ime2 and resulting in elevated sporulation (Fig. 5*E*). The signaling behavior of the Gpa2–10SA mutant is consistent with this model. This mutant displays a diminished level of phosphorylation, reduced translocation to the plasma membrane in response to nitrogen starvation, decreased activation of protein kinase A, and decreased sporulation efficiency. A role of GSKs in promoting sporulation in yeast has been reported (39), and our findings here suggest that Gpa2 may be one of the targets involved.

It has been demonstrated previously that inhibiting the target of rapamycin pathway, either through nitrogen starvation or rapamycin treatment, leads to enhanced activation of protein kinase A (34). Conversely, inhibiting the protein kinase A pathway also elevates the activation level of the target of rapamycin pathway. Thus, there appears to be an antagonistic relationship between these two major nutrient signaling pathways. However, the target that is involved is not known (34). The behavior of Gpa2–10SA suggests that nitrogen starvation–induced phosphorylation of Gpa2 might be one of the underlying mechanisms that allows the antagonistic relationship between the target of rapamycin and protein kinase A pathways. Notably, previous research has indicated that the expression level of Gpr1 is elevated by nitrogen starvation (23), which, presumably, could contribute to the activation of plasma membrane– enriched phosphorylated Gpa2.

Although nitrogen starvation leads to robust phosphorylation of Gpa2, glucose deprivation has very little effect on Gpa2 phosphorylation. A likely reason for that is the different effects of these two nutrient deprivation conditions on the activity of GSKs, as nitrogen starvation, but not glucose deprivation, can lead to an increased activation of GSKs (24, 36). What could be the benefit of phosphorylating Gpa2 under nitrogen starvation conditions but not in response to glucose deprivation? One possibility is that this provides a mechanism that allows cells to distinguish the different nutrient deprivation conditions and modulate the level of protein kinase A activation accordingly. One key function of protein kinase A in yeast is to phosphorylate and stimulate activity of pyruvate kinase, leading to enhanced glycolysis in the presence of glucose (40). Thus, under glucose deprivation conditions, there is probably no need for cells to phosphorylate Gpa2 to boost the activity of protein kinase A. Under nitrogen starvation conditions, however, Gpa2 phosphorylation and the resulting enhanced activation of protein kinase A could allow cells to make better use of glucose, preventing premature cell growth arrest and gaining some advantages.

It remains to be understood how nitrogen starvation and phosphorylation promote plasma membrane enrichment of Gpa2. Our analysis revealed that Plc1 is required for relocalization of Gpa2, suggesting Plc1 as a potential factor that helps retain Gpa2 on plasma membrane. Notably, similar to the Gpa2–10SA mutant, the *plc1*Δ mutant also displays a decreased level of sporulation efficiency (41) and protein kinase A activation (42), consistent with our overall model that plasma membrane enrichment of Gpa2 serves as a mechanism to promote sporulation and protein kinase A activation. It is highly likely that there are other factors that are important for enrichment of Gpa2 on the plasma membrane. One potential way to identify those factors is isolating proteins that interact with Gpa2, and those that differentially interact with Gpa2 in response to nitrogen starvation would be good candidates. Future studies will focus on identifying novel Gpa2 interaction proteins and investigating their roles in regulating Gpa2 localization and signaling.

## Experimental procedures

### *Strains and plasmids*

Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for manipulation of DNA were used throughout. The yeast *S. cerevisiae* strains used in this study were BY4741 (*MAT**a*** *leu2*Δ *met15*Δ *his3*Δ *ura3*Δ), a BY4741-derived mutant lacking *GPA2* (Research Genetics, Huntsville, AL), BY4743 (*MAT*a/α *his3*Δ*1/his3*Δ*1 leu2*Δ*0/leu2*Δ*0 LYS2/lys2*Δ*0 met15*Δ*0/MET15 ura3*Δ*0/ura3*Δ*0*), a BY4743-derived mutant lacking *GPA2* (Dharmacon), a triple-gsk mutant (GSK3-*mck1 rim11 yol128c*, *MAT*α *his3 leu2 ura3 trip1 ade2 mck1::TRP1 rim11:HIS3 yol128c::LEU2*, generously provided by Dr. Claudina Rodrigues-Pousada, Universidade Nova de Lisboa, Portugal) (26), a pka-null mutant GG104 (*MAT**a*** *pde2::TRP1 cdc35::KanMX2 msn2::HIS3 msn4::TRP1*, generously provided by Dr. Joris Winderickx, The Katholieke Universiteit Leuven), and TMY102 (*gpa1::[CFP-GPA1, KAN]*, generously provided by Dr. Tau-Mu Yi) (30). The invasive growth assay was performed in the Σ1278-based invasive strain MLY218a (*MATa leu2 ura3*, from J. Heitman) and MLY2189a-*gpa2* (MLY218a, *gpa2*Δ*::KanMX2*) (this study).

The expression plasmids pYES-Gpa2-FLAG, pYES–Gpa2$^{\Delta 1-120}$–FLAG, pYES-Mck1-FLAG, pYES-Rim11-FLAG, and pYES-Ygk3-FLAG were constructed by amplifying the appropriate DNA fragment using yeast genomic DNA as a template. The individual PCR product was subcloned by digestion with HindIII and ligation to pYES2.1/V5-His-TOPO, which was engineered to have a HindIII site and a C-terminal FLAG tag. The plasmids expressing Gpa2–10SA and Gpa2$^{1-10}$–GFP–Gpa2$^{4-449}$ were constructed by subcloning appropriate DNA fragments synthesized by Integrated DNA Technology to either a pYES vector or pRS315 vector that contains a *GPA2* promoter.

### *Immunoprecipitation and in vitro kinase assay*

The interaction between FLAG-tagged GSK isoforms and Gpa2 was examined using a procedure described in our previous study (43). FLAG-tagged GSK isoforms were purified from yeast cells treated with nitrogen starvation for 12 h to enrich the

activated version of GSK. Cells were lysed in lysis buffer (50 mM $NaPO_4$ (pH 7.5), 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM DTT, 25 mM NaF, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 10 mM *N*-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and 1 pellet of complete EDTA-free protease inhibitor mixture (Roche) for every 50 ml of buffer), and the cleared whole-cell lysate was incubated with anti-FLAG M2 affinity resin (Sigma) for 2 h. After extensive washing with lysis buffer (three times) and kinase buffer (twice; 9802, Cell Signaling Technology), the resin was incubated with FLAG peptide at 30 °C for 30 min to elute the purified FLAG-Mck1 and FLAG-Ygk3. GFP-tagged Gpa2 was isolated from cells in mid-log phase. Cells were lysed using the same lysis buffer as above, and the cleared whole-cell lysate was incubated with anti-GFP magnetic beads (MBL International). After washing extensively with lysis buffer (three times) and kinase buffer (twice), an aliquot of purified FLAG-Mck1 or FLAG-Ygk3 was added to the beads, and ATP was added subsequently (or not) to initiate the kinase reaction. The reaction took place at 30 °C for 3 h, and sample buffer was added to stop the reaction. The kinase reaction was analyzed on 8% Phos-tag SDS-PAGE, and phosphorylated GFP-Gpa2 was detected using anti-GFP (Abcam).

### *Sporulation bioassay*

BY4743-derived *gpa2*Δ cells were transformed with pRS315-$Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$ or pRS315–$Gpa2^{1-10}$–GFP–$Gpa2^{4-449}$–10SA plasmids and grown in synthetic complete dextrose medium at 30 °C overnight. Cell cultures were diluted to $A_{600}$ of about 0.1 and then grown to $A_{600}$ of about 1.8. Cells were collected, washed with sterile water, and resuspended in sporulation medium (2% potassium acetate, 0.005% zinc acetate, 0.02 g/liter uracil, 0.02 g/liter L-histidine, and 0.02 g/liter L-leucine, pH 7.0) to $A_{600}$ of about 1.2. A volume of 2.5 ml of culture was gently mixed on a nutator at 23 °C for 7 days to allow sporulation. The occurrence of sporulation as indicated by ascus appearance was assessed by phase-contrast microscopy.

### *Phosphorylation and immunoblot bioassays*

Gpa2 phosphorylation was analyzed using Phos-tag SDS-PAGE of whole-cell lysates followed by immunoblotting, using antibodies that recognize epitope-tagged Gpa2. For all immunoblot analyses, mid-log phase cells were grown on appropriate medium and treated or not treated with nitrogen starvation or other conditions as indicated. Proteins were extracted via trichloroacetic precipitation, following procedures described previously (27). Whole-cell extracts were resuspended in boiling SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, and 0.0005% bromphenol blue) for 5 min. Following either regular SDS-PAGE or Phos-tag SDS-PAGE and transfer to nitrocellulose, the membrane was probed with antibodies to GFP at 1:5000 (Abcam, ab13970), and FLAG at 1:1000 (Sigma). Immunoreactive species were visualized by enhanced chemiluminescence detection (Pierce) of horseradish peroxidase–conjugated anti-rabbit IgG (Bio-Rad), anti-mouse IgG (Santa Cruz), or anti-Chicken IgY (Abcam). Specificity of detection was established using cell extracts without tagged proteins as negative controls. All experiments were repeated at least three times. Immunoblotting signals were quantified with ImageJ software. The dot bar graphs were generated using Interactive Dotplot (44), and the bars represent standard deviations. Where indicated, the data were statistically analyzed by *t* test, with $p < 0.050$ considered significant.

### *Microscopy analysis*

Cells expressing GFP-tagged Gpa2 or CFP-tagged Gpa1 were grown to either mid-log phase or treated with nitrogen starvation for the indicated times. Cells were concentrated, and 10 μl of concentrated cell suspension was placed on a slide with a thin layer of 0.5% agar and visualized by fluorescence microscopy using an Olympus FV1000 laser-scanning confocal microscope. Fluorescence images were analyzed and quantified with ImageJ software.

### *Sucrose gradient fractionation*

Cells expressing GFP-tagged Gpa2 were subjected to sucrose gradient fractionation using a protocol described previously (29). Spheroplasts were prepared by incubation with 20 μg/ml of zymolyase in SK buffer (1.2 M sorbitol and 0.1 M $KPO_4$ (pH 7.5)) for 45 min at 30 °C. All subsequent steps were carried out at 4 °C. The resulting spheroplasts were washed once with ice-cold SK buffer and resuspended in lysis buffer C (0.8 M sucrose, 20 mM triethanolamine hydrochloride (pH 7.2), 1 mM EDTA, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 1 Roche protease inhibitor mixture tablet per 50 ml) and disrupted by 15 strokes in a motorized homogenizer. The lysate was cleared of unbroken cells with 15 min of 500 × *g* centrifugation. Powdered sucrose was added to each sample to make 70% total sucrose and mixed on a stir plate at 4 °C for 1 h. They were then carefully overlaid with 60%, 50%, 40%, and 30% sucrose solution and subjected to ultracentrifugation at 190,000 × *g* for 19 h. Fifteen equal portions (300 μl) were drawn from the top of each tube, mixed with 2× SDS sample buffer, boiled at 100 °C for 5 min, cooled, and resolved on 10% SDS-PAGE. Blots were probed with anti-GFP to examine GFP-Gpa2 localization. Membrane fraction markers were used to establish which fractions corresponded to which membranes: anti-Ste4 (from Duane Jenness, University of Massachusetts) for the plasma membrane and anti-Pgk1 (from Jeremy Thorner, University of California) for cytosolic fractions.

*Author contributions*—S. H., A. B., R. G., and B. J. data curation; S. H., A. B., R. G., N. C., G. L., B. J., S. K., and Y. W. investigation; S. H., A. B., R. G., B. J., and S. K. methodology; S. H. writing-review and editing; Y. W. conceptualization; Y. W. funding acquisition; Y. W. writing-original draft; Y. W. project administration.

*Acknowledgments—We thank Drs. Claudina Rodrigues-Pousada, Henrik G. Dohlman, Duane Jenness, Jeremy Thorner, and Joris Winderickx for generously providing strains, plasmids, and antibodies.*

### References

1. Zhang, P., Kofron, C. M., and Mende, U. (2015) Heterotrimeric G protein-mediated signaling and its non-canonical regulation in the heart. *Life Sci.* **129,** 35–41 CrossRef Medline

2. Wauson, E. M., Dbouk, H. A., Ghosh, A. B., and Cobb, M. H. (2014) G protein-coupled receptors and the regulation of autophagy. *Trends Endocrinol. Metab.* **25,** 274–282 CrossRef Medline
3. Gainetdinov, R. R., Premont, R. T., Bohn, L. M., Lefkowitz, R. J., and Caron, M. G. (2004) Desensitization of G protein-coupled receptors and neuronal functions. *Annu. Rev. Neurosci.* **27,** 107–144 CrossRef Medline
4. Sato, M. (2013) Roles of accessory proteins for heterotrimeric G-protein in the development of cardiovascular diseases. *Circ. J.* **77,** 2455–2461 CrossRef Medline
5. Heng, B. C., Aubel, D., and Fussenegger, M. (2013) An overview of the diverse roles of G-protein coupled receptors (GPCRs) in the pathophysiology of various human diseases. *Biotechnol. Adv.* **31,** 1676–1694 CrossRef Medline
6. Lombardi, M. S., Kavelaars, A., and Heijnen, C. J. (2002) Role and modulation of G protein-coupled receptor signaling in inflammatory processes. *Crit. Rev. Immunol.* **22,** 141–163 Medline
7. Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J. H., Gorwa, M. F., Colavizza, D., and Thevelein, J. M. (1998) Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **17,** 3326–3341 CrossRef Medline
8. Nazarko, V. Y., Thevelein, J. M., and Sibirny, A. A. (2008) G-protein-coupled receptor Gpr1 and G-protein Gpa2 of cAMP-dependent signaling pathway are involved in glucose-induced pexophagy in the yeast *Saccharomyces cerevisiae*. *Cell Biol. Int.* **32,** 502–504 CrossRef Medline
9. Kraakman, L., Lemaire, K., Ma, P., Teunissen, A. W., Donaton, M. C., Van Dijck, P., Winderickx, J., de Winde, J. H., and Thevelein, J. M. (1999) A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* **32,** 1002–1012 CrossRef Medline
10. Peeters, T., Louwet, W., Geladé, R., Nauwelaers, D., Thevelein, J. M., and Versele, M. (2006) Kelch-repeat proteins interacting with the Gα protein Gpa2 bypass adenylate cyclase for direct regulation of protein kinase A in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 13034–13039 CrossRef Medline
11. Versele, M., de Winde, J. H., and Thevelein, J. M. (1999) A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. *EMBO J.* **18,** 5577–5591 CrossRef Medline
12. Donzeau, M., and Bandlow, W. (1999) The yeast trimeric guanine nucleotide-binding protein alpha subunit, Gpa2p, controls the meiosis-specific kinase Ime2p activity in response to nutrients. *Mol. Cell Biol.* **19,** 6110–6119 CrossRef Medline
13. Swaney, D. L., Beltrao, P., Starita, L., Guo, A., Rush, J., Fields, S., Krogan, N. J., and Villén, J. (2013) Global analysis of phosphorylation and ubiquitylation cross-talk in protein degradation. *Nat. Methods* **10,** 676–682 CrossRef Medline
14. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425,** 686–691 CrossRef Medline
15. Kinoshita, E., Kinoshita-Kikuta, E., Kubota, Y., Takekawa, M., and Koike, T. (2016) A Phos-tag SDS-PAGE method that effectively uses phosphoproteomic data for profiling the phosphorylation dynamics of MEK1. *Proteomics* **16,** 1825–1836 CrossRef Medline
16. Horinouchi, T., Terada, K., Higashi, T., and Miwa, S. (2016) Using Phos-Tag in western blotting analysis to evaluate protein phosphorylation. *Methods Mol. Biol.* **1397,** 267–277 CrossRef Medline
17. Dratz, E. A., Furstenau, J. E., Lambert, C. G., Thireault, D. L., Rarick, H., Schepers, T., Pakhlevaniants, S., and Hamm, H. E. (1993) NMR structure of a receptor-bound G-protein peptide. *Nature* **363,** 276–281 CrossRef Medline
18. Hamm, H. E., Deretic, D., Arendt, A., Hargrave, P. A., Koenig, B., and Hofmann, K. P. (1988) Site of G protein binding to rhodopsin mapped with synthetic peptides from the α subunit. *Science* **241,** 832–835 CrossRef Medline
19. Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caffrey, M., Gellman, S. H., *et al.* (2011) Crystal structure of the β2 adrenergic receptor-$G_s$ protein complex. *Nature* **477,** 549–555 CrossRef Medline
20. Harashima, T., and Heitman, J. (2005) Gα subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP-induced dimorphic transitions in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **16,** 4557–4571 CrossRef Medline
21. Stone, D. E., Cole, G. M., de Barros Lopes, M., Goebl, M., and Reed, S. I. (1991) *N*-myristoylation is required for function of the pheromone-responsive Gα protein of yeast: conditional activation of the pheromone response by a temperature-sensitive *N*-myristoyl transferase. *Genes Dev.* **5,** 1969–1981 CrossRef Medline
22. Dohlman, H. G., Goldsmith, P., Spiegel, A. M., and Thorner, J. (1993) Pheromone action regulates G-protein α-subunit myristoylation in the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 9688–9692 CrossRef Medline
23. Lorenz, M. C., Pan, X., Harashima, T., Cardenas, M. E., Xue, Y., Hirsch, J. P., and Heitman, J. (2000) The G protein-coupled receptor gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Genetics* **154,** 609–622 Medline
24. Xiao, Y., and Mitchell, A. P. (2000) Shared roles of yeast glycogen synthase kinase 3 family members in nitrogen-responsive phosphorylation of meiotic regulator Ume6p. *Mol. Cell Biol.* **20,** 5447–5453 CrossRef Medline
25. Jin, X., Starke, S., Li, Y., Sethupathi, S., Kung, G., Dodhiawala, P., and Wang, Y. (2016) Nitrogen starvation-induced phosphorylation of Ras1 protein and its potential role in nutrient signaling and stress response. *J. Biol. Chem.* **291,** 16231–16239 CrossRef Medline
26. Pereira, J., Pimentel, C., Amaral, C., Menezes, R. A., and Rodrigues-Pousada, C. (2009) Yap4 PKA- and GSK3-dependent phosphorylation affects its stability but not its nuclear localization. *Yeast* **26,** 641–653 CrossRef Medline
27. Slessareva, J. E., Routt, S. M., Temple, B., Bankaitis, V. A., and Dohlman, H. G. (2006) Activation of the phosphatidylinositol 3-kinase Vps34 by a G protein α subunit at the endosome. *Cell* **126,** 191–203 CrossRef Medline
28. Lo, I. C., Gupta, V., Midde, K. K., Taupin, V., Lopez-Sanchez, I., Kufareva, I., Abagyan, R., Randazzo, P. A., Farquhar, M. G., and Ghosh, P. (2015) Activation of $G\alpha_i$ at the Golgi by GIV/Girdin imposes finiteness in Arf1 signaling. *Dev. Cell* **33,** 189–203 CrossRef Medline
29. Song, J., Hirschman, J., Gunn, K., and Dohlman, H. G. (1996) Regulation of membrane and subunit interactions by *N*-myristoylation of a G protein α subunit in yeast. *J. Biol. Chem.* **271,** 20273–20283 CrossRef Medline
30. Yi, T. M., Kitano, H., and Simon, M. I. (2003) A quantitative characterization of the yeast heterotrimeric G protein cycle. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 10764–10769 CrossRef Medline
31. Xue, Y., Batlle, M., and Hirsch, J. P. (1998) GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Gα subunit and functions in a Ras-independent pathway. *EMBO J.* **17,** 1996–2007 CrossRef Medline
32. Ansari, K., Martin, S., Farkasovsky, M., Ehbrecht, I. M., and Küntzel, H. (1999) Phospholipase C binds to the receptor-like GPR1 protein and controls pseudohyphal differentiation in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **274,** 30052–30058 CrossRef Medline
33. Thevelein, J. M., and de Winde, J. H. (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **33,** 904–918 CrossRef Medline
34. Ramachandran, V., and Herman, P. K. (2011) Antagonistic interactions between the cAMP-dependent protein kinase and Tor signaling pathways modulate cell growth in *Saccharomyces cerevisiae*. *Genetics* **187,** 441–454 CrossRef Medline
35. Zeller, C. E., Parnell, S. C., and Dohlman, H. G. (2007) The RACK1 ortholog Asc1 functions as a G-protein β subunit coupled to glucose responsiveness in yeast. *J. Biol. Chem.* **282,** 25168–25176 CrossRef Medline
36. Broach, J. R. (2012) Nutritional control of growth and development in yeast. *Genetics* **192,** 73–105 CrossRef Medline
37. Yoshida, M., Kawaguchi, H., Sakata, Y., Kominami, K., Hirano, M., Shima, H., Akada, R., and Yamashita, I. (1990) Initiation of meiosis and sporulation in *Saccharomyces cerevisiae* requires a novel protein kinase homologue. *Mol. Gen. Genet.* **221,** 176–186 Medline
38. Mitchell, A. P., Driscoll, S. E., and Smith, H. E. (1990) Positive control of sporulation-specific genes by the IME1 and IME2 products in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* **10,** 2104–2110 CrossRef Medline

39. Neigeborn, L., and Mitchell, A. P. (1991) The yeast MCK1 gene encodes a protein kinase homolog that activates early meiotic gene expression. *Genes Dev.* **5,** 533–548 CrossRef Medline
40. Portela, P., Howell, S., Moreno, S., and Rossi, S. (2002) *In vivo* and *in vitro* phosphorylation of two isoforms of yeast pyruvate kinase by protein kinase A. *J. Biol. Chem.* **277,** 30477–30487 CrossRef Medline
41. Flick, J. S., and Thorner, J. (1998) An essential function of a phosphoinositide-specific phospholipase C is relieved by inhibition of a cyclin-dependent protein kinase in the yeast *Saccharomyces cerevisiae*. *Genetics* **148,** 33–47 Medline
42. Demczuk, A., Guha, N., Nguyen, P. H., Desai, P., Chang, J., Guzinska, K., Rollins, J., Ghosh, C. C., Goodwin, L., and Vancura, A. (2008) *Saccharomyces cerevisiae* phospholipase C regulates transcription of Msn2p-dependent stress-responsive genes. *Eukaryot. Cell* **7,** 967–979 CrossRef Medline
43. Li, Y., and Wang, Y. (2013) Ras protein/cAMP-dependent protein kinase signaling is negatively regulated by a deubiquitinating enzyme, Ubp3, in yeast. *J. Biol. Chem.* **288,** 11358–11365 CrossRef Medline
44. Weissgerber, T. L., Savic, M., Winham, S. J., Stanisavljevic, D., Garovic, V. D., and Milic, N. M. (2017) Data visualization, bar naked: a free tool for creating interactive graphics. *J. Biol. Chem.* **292,** 20592–20598 CrossRef Medline