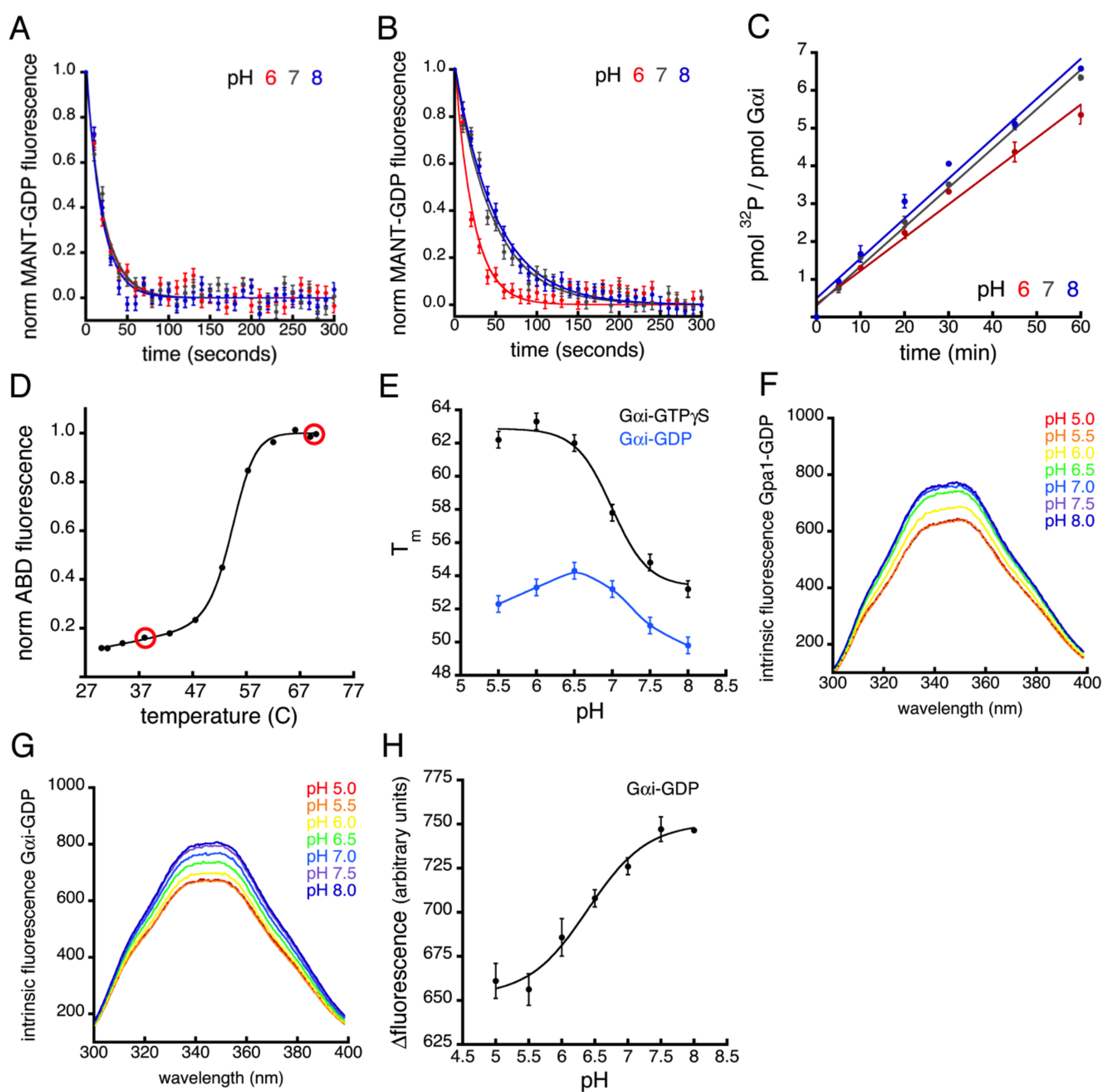

Figure S1. Biophysical analysis of representative Gα subunits, related to Figure 2. pH dependence of nucleotide exchange by yeast Gα (**A**) and mammalian Gαi (**B**) measured using the fluorescent nucleotide analog MANT-GDP. (**C**) Steady-state GTP hydrolysis by mammalian Gαi at pH 6.0, 7.0, and 8.0. (**D**) A representative fast quantitative cysteine reactivity (fQCR) unfolding curve for mammalian Gαi at pH 7.0. Red circles indicate the conditions for which the pH dependence of cysteine labeling was probed by fQCR and quantified by mass spectrometry (Figure 2D-E). (**E**) The pH-

dependent thermal stability of GTPγS- and GDP-bound mammalian Gαi measured by fQCR. (**F, G**) Intrinsic tryptophan fluorescence spectra of yeast (**F**) and mammalian (**G**) Gα as a function of pH. (**H**) Intrinsic tryptophan fluorescence of GDP-bound mammalian Gαi (at 345 nm) as a function of pH. Error bars represent ± standard deviation of the normalized fluorescence signal (panels **A** and **B**), and ± SEM of three (panels **C** and **E**) or four (panel **H**) independent experiments.

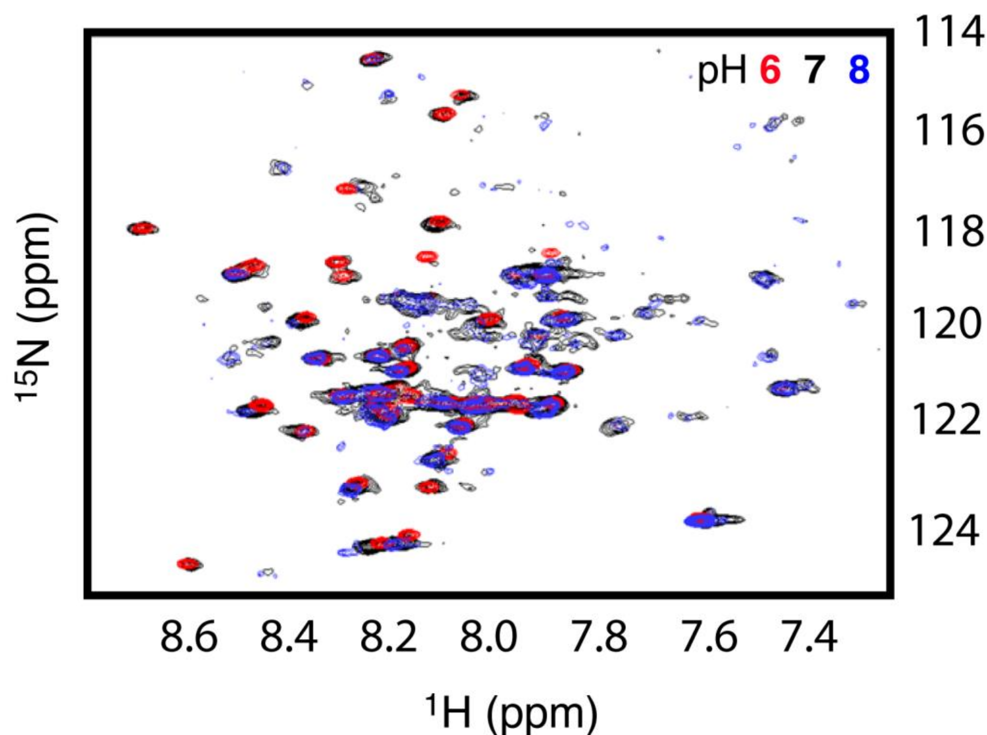

Figure S2. pH dependence of Gα conformation revealed by NMR, related to Figure 2. Detail of the overlaid heteronuclear single quantum coherence spectra of Gαi collected at pH 6, 7, and 8. Although the majority of the peaks in these three spectra overlap, there are distinct peaks that are common only to the spectra at pH 6 and 7, and pH 7 and 8. These differences indicate that Gαi adopts distinct backbone conformations at pH 6 and 8 that exist in a mixed equilibrium at pH 7. For NMR studies, ^{15}N-enriched samples of Gαi were generated using standard protocols in M-9 minimal media. Gαi in PBS-GMT was concentrated to 200 μM in the presence of 10 % D_2O and supplemented with 1 mM GTPγS and 0.5 mM $MgCl_2$. ^{1}H-^{15}N 2D HSQC experiments were conducted on a Varian 700 MHz with a cryoprobe at 30°C. All NMR data were processed and analyzed using NMRPipe and NMRDraw as well as NMRViewJ software. Despite the challenges

associated with NMR experiments on large proteins, we were able to observe amide chemical shifts for approximately one-third of the Gαi backbone at all three pH values.

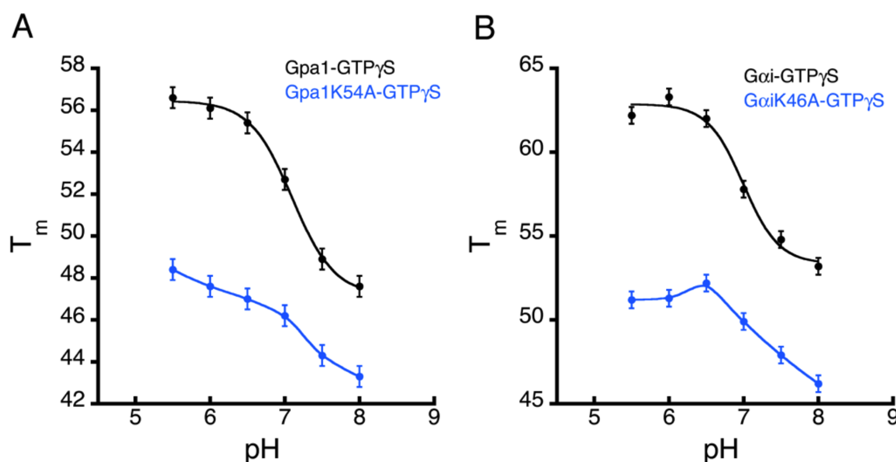

Figure S3. Mutations of core network components alter the pH-dependent stability of yeast (**A**) and mammalian (**B**) Gα, related to Figure 2. Here we show representative thermostability data for the alanine substitution of a core network lysine conserved in both Gpa1 (K54) and mammalian Gαi (K46). As expected, both lysine mutations are highly destabilized at all pH values. This loss in stability can be described as having two separate components. The first is a down-shift in the mutant thermostability profile on the y-axis. This shift is due to a reduction in stability caused by pH-independent mechanisms such as changes in sterics and packing, changes in hydrogen bonding, and the potential generation of uncompensated charges on sidechains within the core network, on the phosphates of the GTPγS ligand, or both. The second descriptive component of the pH profile is a pH-dependent loss in G protein themostability from pH 5.5 to 8.0. It is this component of the stability profile that reflects the influence of shifted pK_a values and serves as the basis for assessing a change in pH-sensitivity (i.e. the ability to sense pH). In the case of the mutant pH profiles, the reduced magnitude change (indicated by a pH-dependent reduction in ΔT_m from ~10 to 4) and cooperativity (indicated by a reduction in

Hill coefficient from 2 to < 1) is consistent with a dramatic disruption to Gpa1 and Gαi pH sensing. Error bars represent ± SEM of three independent experiments.

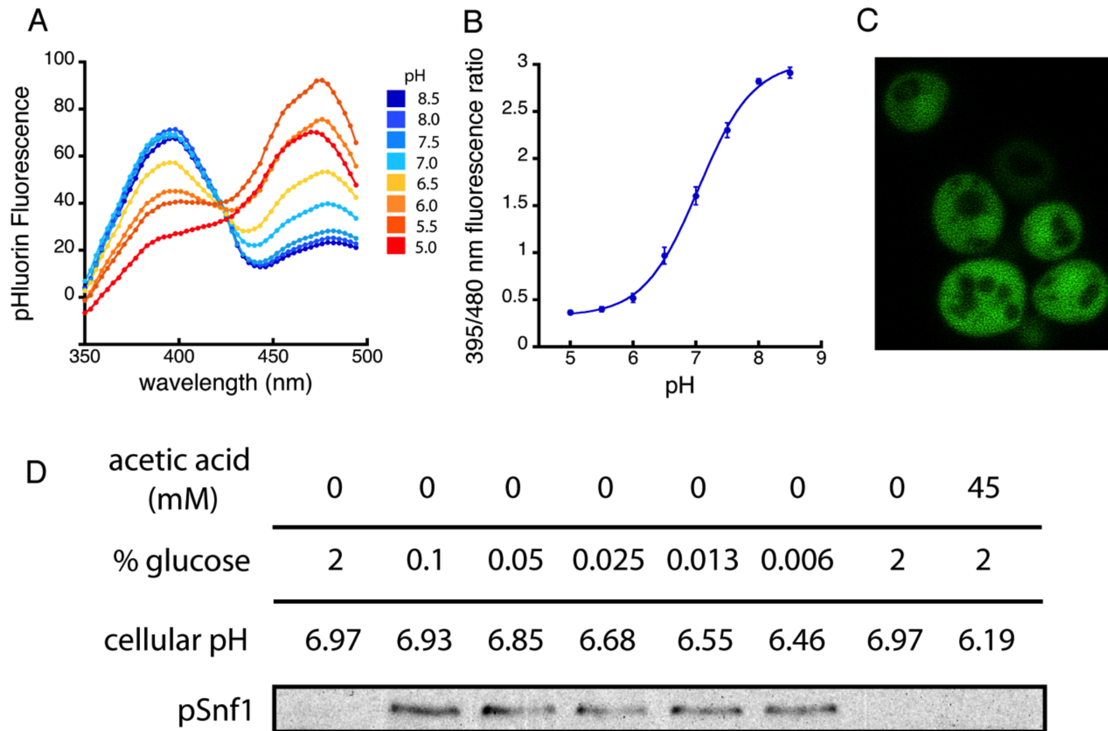

Figure S4. Calibration of the genetically-encoded pH sensor pHluorin, related to Figures 3 and 4. (**A**) pHluorin spectra of yeast that were permeabilized and resuspended in a panel of calibration buffers at different pH. (**B**) The ratio of fluorescence intensities for the neutral (395 nm) and anionic (480 nm) pHluorin flourophore in panel **A** were plotted versus pH to generate a standard curve. The solid blue line represents a fit of the modified-Hill equation (Equation 1) to the data. Error bars represent ± SEM of four independent experiments. (**C**) Images obtained by confocal microscopy demonstrated that the pHluorin probe was distributed homogeneously throughout the yeast cytoplasm. (**D**) The activity of the AMP-kinase (Elm1), which is responsible for phosphorylating both Gpa1 and the nutrient-sensing kinase Snf1, was unaffected by changes in cellular pH. Snf1 is phosphorylated (pSnf1) in response to low glucose (<0.1%) over a range of cellular pH values. Unlike Gpa1 (Figures 3D-E, 4A-B), Snf1 was not phosphorylated

under conditions of high glucose (2%), even when cellular pH was lowered to 6.19 using acetic acid.

Supplemental Experimental Procedures

Protein production

Proteins were overexpressed in *E. coli* (BL21 RIPL strain) using the method of auto-induction. Starter cultures in LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 µg/µL carbenicillin) were inoculated with freshly transformed bacterial colonies and grown overnight at 37°C. The following day the entire starter culture was used to inoculate auto-induction media: 800 mL of autoclaved ZY media (10 g/L tryptone, 5 g/L yeast extract), 16 mL of sterile-filtered 50 x 5052 solution (25% w/v glycerol, 2.5% w/v glucose, 10% w/v α-lactose), 16 mL of sterile-filtered 50 x M buffer (1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4CL, 0.25 M Na_2SO_4), 1.6 mL of 1 M $MgSO_4$, and 800 µL of 50 µg/µL carbenicillin in 50% EtOH. The cultures were grown at 37°C for 8 hours to achieve a typical A_{600} between 2 and 4. The temperature was then reduced to 18°C and the cultures were left to grow overnight. After 16 hours of growth the bacteria were harvested by centrifugation and resuspended in 50 mL PBS (25 mM potassium phosphate, 100 mM KCl, pH 7.0). The resuspended bacteria were then frozen at -20°C. Overexpressed Gpa1 (UniProt identifier P08539, residues 37 to 435) and mammalian Gαi (UniProt identifiers B2RSH2, P63096, P63097) containing C-terminal His-tags (6X) were batch-purified on ice using HIS-Select® Nickel Affinity Gel (Sigma-Aldrich Aldrich, P6611). Frozen bacteria were thawed, TCEP was added (to 1 mM final), and cells were lysed by French press (one pass at ~1,000 psi) in PBS supplemented with 50 µM GDP, 50 µM $MgCl_2$, and 1 mM TCEP (PBS-GMT). Samples were kept at 4°C throughout the purification. Clarified lysate (13 mL) was combined with 50% affinity gel slurry (1 mL) in a 15 mL conical vial and gently mixed end-over-end for 15 minutes.

The affinity gel was collected by centrifugation and washed in 13 mL of PBS-GMT. This procedure was repeated two more times for a total of three washes. Protein was eluted by transferring a 2 mL slurry of protein-bound affinity gel to a 3 mL fritted-spin column (Thermo Scientific, 89896), and excess buffer was drained by gravity flow. The spin column was then capped, 2 mL of elution buffer (PBS-GMT with 250 mM imidazole) was added, and the column was closed and gently mixed end-over-end for 15 minutes. The eluted protein sample was then transferred to a 3 mL Slide-A-Lyzer® cassette (Thermo Scientific, 66330) and dialyzed against 4 L of PBS-GMT for 1 hour. The dialysis cassette was then transferred to 4 L of fresh PBS-GMT and dialyzed overnight. Following dialysis, the purified protein was transferred to 1.5 mL tubes and stored at 4°C. This procedure typically yielded 2 mL of protein at a concentration between 50 and 100 μM. All biochemical and biophysical experiments in this study were done using purified protein that was no more than three days old (post dialysis), and never frozen.

pH dependence of nucleotide exchange

Protein stock (in PBS-GMT) was diluted to 0.2 μM in PBS (pH 6, 7, or 8) supplemented with 50 μM $MgCl_2$, and 0.5 μM 2'-(or-3')-*O*-(*N*-Methylanthraniloyl) Guanosine 5'-Diphosphate (MANT-GDP) (Molecular Probes, M-12414). To ensure the Gα subunits were fully loaded with MANT-GDP, samples were incubated in a quartz cuvette at ambient room temperature for 20 minutes. MANT-GDP release was initiated by adding excess GDP (1 μM), and quantified in a Perkin Elmer LS55 Spectrometer using excitation and emission wavelengths of 280 and 440 nm. The data reported in Figure S1 correspond to the mean (values) and standard deviation (error bars) of two experiments done at each of the indicated pH values.

pH dependence of protein thermostability

Protein stock (in PBS-GMT) was diluted to 1 µM in cold PBS (pH 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0) with 50 µM GDP or GTPγS and chilled on ice. For each unfolding reaction, 5 µL of 26 mM working ABD stock solution (1 mM final) was combined with 125 µL of ice-cold protein sample. For unfolding reactions done at or below pH 6.0, the volume of working ABD stock solution was doubled, giving a final ABD concentration of 2 mM. Samples were mixed on ice by pipetting up and down five times, and 10 µL aliquots were distributed across a 12-well PCR strip-tube (USA Scientific, 1402-2408), also chilled on ice. The samples were then placed in a gradient thermocycler (Biometra TProfessional Thermocycler), heated for 3 minutes, immediately transferred to ice, and quenched by adding 2 µL of 0.1N HCl—a reduction in temperature or pH dramatically slows the ABD-labeling reaction. The quenched samples were then transferred to a 384-well plate (Greiner, 788076) and their fluorescence values were quantified in a BMG Labtech PHERAstar plate reader using excitation and emission bandpass filters of 400 and 500 nm. The resultant thermal unfolding curves were fit using a two-state model of protein unfolding to provide values for the midpoint of thermal unfolding (T_m). Thermostability experiments were repeated multiple times to established a mean T_m uncertainty of ±0.5°C (error bars in Figure 2B and S1). In the case of GTPγS-bound Gα subunits, the solid lines in Figure 2B and S1 represent a fit of the modified Hill equation to the pH-dependent T_m values (Equation 1),

$$T_m(pH) = \left(b_h - b_l\right)\left(\frac{10^{n\left(pH - pK_a\right)}}{1 + 10^{n\left(pH - pK_a\right)}}\right) + b_l \qquad \textbf{(1)}$$

where β_h and β_l are the higher and lower baselines, n is the Hill coefficient, and pK_a is the apparent pK_a value of the core network.

Quantitative mass spectrometry of ABD-labeled Gα subunits

Gαi stock (in PBS-GMT) was diluted to 3 μM in PBS (pH 6.0 or 7.0) supplemented with 50 μM GDP and incubated on ice. 1.2 mL of protein sample was mixed with 100 μL of 26 mM working ABD stock solution (final ABD concentration of 2 mM), and 20 μL aliquots of this reaction mixture were distributed across an 8-well PCR strip-tube chilled on ice. Four sample sets— pH 6.0 at 42°C and 70°C, pH 7.0 at 42°C and 70°C—were reacted for three minutes and transferred to ice to quench the labeling reaction. Each sample set was consolidated into a single tube (15 μg per condition), and the unreacted ABD was removed using a spin ultrafiltration unit with a molecular weight cutoff of 30 kDa (Sartorius Stedim Biotech.).

To prepare the ABD-labeled proteins for mass spectrometry, each set of Gαi samples was reduced, alkylated, and digested using the FASP protocol with the following modifications: 20 mM TCEP was used instead of DTT, and TEAB buffer (100 mM triethylammonium bicarbonate, pH 8.5) was used in place of TRIS buffer. The peptide solutions were lyophilized, resuspended in TEAB buffer, and differentially labeled with the 4-plex iTRAQ reagent: one iTRAQ reporter for each of the four labeling conditions. The 4 sets of iTRAQ-labeled peptides were then mixed and loaded onto a 2 cm long X 360 μm o.d. × 100 μm i.d. microcapillary fused silica precolumn packed with Magic 5 μm C18AQ resin (Michrom Biosciences, Inc.) for LC-MS/MS analysis. After sample loading the precolumn was washed with a mixture of 95% Solvent A (0.1% formic acid in water) and 5% Solvent B (0.1% formic acid in acetonitrile) for 20 min at a flow rate of 2 μL/min. The precolumn was then connected to a 360 μm o.d. × 75 μm i.d. analytical column packed with 14 cm of 5 μm C18 resin constructed with an integrated electrospray

emitter tip. The peptides were eluted at a flow rate of 250 nL/min by increasing the percentage of solvent B to 40% with a Nano-Acquity HPLC solvent delivery system (Waters Corp.). The LC system was directly connected through an electrospray ionization source interfaced to an LTQ Orbitrap Velos ion trap mass spectrometer (Thermo Electron Corp.). The mass spectrometer was controlled by Xcalibur software (Thermo., ver. 2.1.0.1140) and operated in the data-dependent mode in which the initial MS scan recorded the mass to charge (m/z) ratios of ions over the range 400–2000. The 5 most abundant ions were automatically selected for subsequent CID (collision-activated dissociation) and HCD (higher energy collision-induced dissociation). Raw files were searched using MASCOT (Matrix Science, Ver. 2.3.02) via Proteome Discoverer (Thermo Scientific, Ver. 1.3.0.339) against the sequence for mammalian Gαi (UniProt identifiers B2RSH2, P63096, P63097). Search parameters included peptide mass tolerance of 10 ppm, fragment ion tolerance of 0.8 mass unit, variable modifications for methionine oxidation and ABD or IAA-labeling of cysteine residues, and iTRAQ labeling of peptide N-termini, Lys, and Tyr residues. Peptides with an Expectation Value of less than 0.01 were considered "high confidence". Using this procedure we were able to reproducibly identify sets of labeled peptides representing 4 of the 9 cysteines in Gαi (see Figure 2E).

Relative ABD labeling (Figure 2E) was quantified by a three-step procedure. First, the ratios of the four iTRAQ reporter ions—corresponding to Gαi peptides labeled at pH 6.0 (42°C), pH 6.0 (70°C), pH 7.0 (42°C), and pH 7.0 (70°C)—were normalized to the most abundant iTRAQ label. This correction was minor (1-2%), given that the total peptide concentration was nearly identical across the four sample sets prior to mixing.

Second, fractional ABD-labeling (θ) was calculated at each pH (6.0 and 7.0) as the ABD-labeling ratio (R) of each cysteine at low (42°C) and high (70°C) temperature (i.e. $\theta_{pH} = R_{42°C}/R_{70°C}$). Third, the set of relative ABD labeling values (Figure 2D) was calculated for each pH (6.0 and 7.0) by normalizing to the minimum θ_{pH} value (i.e. the θ_{pH} value for Cys139 at pH 6.0, and Cys254 at pH 7.0). The data in Figure 2D represent the mean (values) and standard deviation (error bars) derived from box-whisker plots (Wolfram Mathematica, 8.0.0.0) of four consolidated cysteine-protection experiments (two independent experiments, each with two analytical repeats). The combined dataset consisted of 586 peptide observations (Cys66 n=232, Cys139 n=107, Cys254 n=98, Cys325 n=149).

In vivo quantification of intracellular pH and GPCR signaling

Yeast strains and media. Standard methods for growth, maintenance, and transformation of yeast were employed throughout. Except where indicated otherwise, all experiments were done in SDC-pH5 medium (or SDC-pH5 lacking the indicated nutrient) composed of 50 mM dibasic potassium phosphate, 50 mM dibasic sodium succinate, 2% glucose, 1.7 g/L yeast nitrogen base, 30 mg/L adenine, 5 g/L ammonium sulfate, 0.79 g/L CSM mixture (MP Biomedicals), titrated to pH 5.0 with HCl, and sterile filtered. Strains used in this study were BY4741 (*MAT***a** leu2Δ met15Δ his3Δ ura3Δ) and BY4741-derived mutants: *ste4*Δ::KanMX4 from the yeast deletion library (Invitrogen), $TetO_7$-WT and $TetO_7$-Pma1 from the yeast Tet-promoter Hughes Collection (yTHC, Open Biosystems), and *sst2*Δ::KanMX4 was re-made in this laboratory. To measure intracellular pH, yeast strains were transformed with the pYEplac181 plasmid (2μ, amp^R, *LEU2*$^+$) containing the pHluorin gene under control of a *TEF1* promoter (a gift from Rajini Rao, Johns Hopkins University).

pHluorin calibration. 9 mL of yeast culture (grown to an A_{600nm} of 0.8) was combined with a 1 mL of digitonin (Sigma-Aldrich, D141) solution (1 mg/mL in PBS, pH 7.0) in a 10 mL vial, vortex-mixed, and incubated with shaking at 30°C for 10 minutes. After digitonin permeabilization, yeast cells were harvested by centrifugation, washed with 25 mL of PBS (pH 7.0), harvested again, and resuspended in 25 mL of fresh PBS (pH 7.0). One mL of washed and permeabilized yeast cells was then distributed to eight 1.5 mL tubes. The cells in each tube were harvested by centrifugation, cleared of supernatant, and resuspended in 1 mL of PBS (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) or Tris buffer (25 mM Tris, 100 mM KCl, pH 8.5). 300 μL of each sample was then dispensed

(in triplicate) into a 96-well plate (BD Falcon, 351172), and the pHluorin spectra were measured in a fluorescence plate reader (SpectraMax M5 by Molecular Devices). The ratio of fluorescence intensities for the neutral (395 nm) and anionic (480 nm) pHluorin flourophore (*r*) was plotted versus pH (Figure S3) to construct a standard curve described mathematically by the modified-Hill equation (Equation 1),

$$r(pH) = (\beta_h - \beta_l)\left(\frac{10^{n(pH - pKa)}}{1 + 10^{n(pH - pKa)}}\right) + \beta_l \qquad (\mathbf{1})$$

where β_h and β_l are the higher and lower baselines, *n* is the Hill coefficient, and pK_a is the apparent pK_a value of the pHluorin fluorophore. After algebraic rearrangement, Equation 1 was used to convert pHluorin fluorescence ratios to values of cellular pH (Equation 2),

$$pH = pK_a + n^{-1} \log \frac{\left(\frac{r - \beta_l}{\beta_h - \beta_l}\right)}{\left(1 - \frac{r - \beta_l}{\beta_h - \beta_l}\right)} \qquad (\mathbf{2})$$

where β_h was 3.0362, β_l was 0.32701, *n* was 0.99673, and pK_a was 7.0444 (i.e. the parameters obtained by a least squares fit of the function *r*(pH) to the data presented in Figure S3). The standard curve data in Figure S3 represent the mean (values) and standard deviation (error bars) of four experiments.

Manipulation of intracellular pH. For experiments in which acetic acid was used to manipulate intracellular pH (Figure 3B), yeast cultures where grown overnight at 30°C to an A_{600nm} of 0.9. A 10 mL aliquot of this culture was then transferred to a 50 mL conical tube, and the cells were harvested by centrifugation and resuspended in 10 mL of fresh media. Dose-dependent pH time courses were collected via a three-step procedure. First a row of a 96-well microplate (BD Falcon, 351172) was pre-loaded with sample blanks for each experimental condition (20 µL of 10X acetic acid dose and 280 µL of media). Second, the next two rows of the plate were pre-loaded with 20 µL volumes of 10X acetic acid (dose-row) and 300 µL of yeast culture (culture-row), respectively. Third, using a multichannel pipette, 280 µL from culture-row was added to the dose-row and mixed by pipette (6 times). The microplate was then immediately transferred to the plate reader and sample (and blank) fluorescence values were measured for 90 minutes (every 30 seconds) at ambient room temperature.

For experiments in which glucose was used to manipulate intracellular pH (Figure 3A), yeast cultures where grown overnight at 30°C to an A_{600nm} of 0.9. A 10 mL aliquot of culture was then transferred to a 50 mL conical tube, and the cells were harvested by centrifugation, washed with 25 mL of glucose-free media, harvested by centrifugation again, and resuspended in 10 mL of glucose-free media. Dose-dependent pH time courses were collected using 10X glucose doses and the three-step procedure described above for acetic acid treatment.

For experiments in which gene knockdown of the proton pump Pma1 was used to manipulate cellular pH (Figure 3C), yeast cultures were grown in selective media (-Uracil) overnight at 30°C to an A_{600nm} of 0.8 with or without 10 µg/mL doxycycline

(Sigma-Aldrich, D9891). For the TetO$_7$-Pma1 strain, which grew slowly after doxycycline treatment, overnight culture was harvested by centrifugation, resuspended in media containing fresh doxycycline, and grown to an A_{600} of 0.8. The cellular pH of each yeast strain (with or without doxycycline treatment) was quantified for 300 µL sample aliquots (in triplicate) in the fluorescence microplate reader at ambient room temperature.

Proper background subtraction was a critical factor determining the accuracy and reproducibility of our intracellular pH measurements. Sample blanks must comprise the identical media (including any supplemental reagents) and volume of the experimental cultures being tested. In all experiments the background corrected fluorescence ratios reported by the pHluorin probe were converted to cellular pH using Equation 2. For all pH measurements, the reported pH values correspond to the mean (values) and standard deviation (error bars) of at least three experimental repeats.

Gpa1 phosphorylation. The data in Figure 3D correspond to yeast cultures grown in selective media (-Uracil) overnight at 30°C to an A_{600nm} of 0.7 with or without 10 µg/mL doxycycline. Cells were then harvested by centrifugation, washed in low-glucose (0.05%) media, resuspended in low- (0.05%) or high-glucose (2%) media, incubated at ambient room temperature for 5 minutes (without shaking), quenched with trichloroacetic acid (TCA) (2% final), and immediately chilled on ice. The quenched samples were then harvested by centrifugation, resuspended in 1 mL of 10 mM sodium azide solution, transferred to a 1.5 mL tube, harvested again, supernatant was removed, pellets were frozen at -80°C, and analyzed using the western blotting procedure described below.

For the data in Figure 3E, yeast cultures were grown overnight at 30°C to an A_{600nm} of 0.9, harvested by centrifugation, washed with fresh media, harvested again by centrifugation, resuspended in fresh media, and combined with a 10X dose of acetic acid (450 mM) to a final A_{600nm} of 0.8. Samples were incubated at ambient room temperature for 10 minutes (without shaking), quenched with TCA (2% final), and immediately chilled on ice. The quenched samples were then harvested by centrifugation, resuspended in 1 mL of 10 mM sodium azide solution, transferred to a 1.5 mL tube, harvested again, supernatant was removed, pellets were frozen at -80°C, and analyzed using the western blotting procedure described below.

pH dependence of Gpa1 and MAPK phosphorylation. For the data in Figure 4, 100 mL yeast cultures where grown overnight at 30°C to an A_{600nm} of 0.9. Aliquots (25 mL) were then split across four 50 mL conical vials, harvested by centrifugation, resuspended in fresh media, and kept at ambient room temperature for the remainder of the experiment. A panel of 50 mL conical vials was then pre-loaded with 1 mL of 10X acetic acid dose (0, 100, 200, 300, 350, 400, and 450 mM) and 10 µL of 1000X α-factor (300 µM). Pheromone signaling and intracellular pH dynamics were initiated simultaneously by adding 9 mL of yeast culture to each pre-loaded tube (at 30 second offsets), and vortex-mixed for five seconds. Samples were incubated at ambient room temperature for 10 minutes (without shaking), quenched by adding 200 µL of TCA (2% final), and immediately chilled on ice. The quenched samples were then harvested by centrifugation, resuspended in 1 mL of 10 mM sodium azide solution, transferred to a 1.5 mL tube, harvested again, supernatant was removed, pellets were frozen at -80°C, and analyzed using the western blotting procedure described below. The data presented in

Figures 4 (panels A, D, F) correspond to the mean (values) and standard deviation (error bars) of at least three experiments.

Snf1 phosphorylation. For the data in Figure S3, 100 mL yeast cultures where grown overnight at 30°C to an A_{600nm} of 0.9. Aliquots (25 mL) were then split across four 50 mL conical vials, harvested by centrifugation, washed with 25 mL of glucose-free media, resuspended in 25 mL of glucose-free media, and kept at ambient room temperature for the remainder of the experiment. A panel of 50 mL conical vials was then pre-loaded with 1 mL of 10X glucose and acetic acid doses. Intracellular nutrient and pH dynamics were initiated simultaneously by adding 8 mL of yeast culture to each pre-loaded tube (at 30 second offsets), and vortex-mixed for five seconds. Samples were incubated at ambient room temperature for 10 minutes (without shaking), quenched by adding 200 µL of TCA (2% final), and immediately chilled on ice. The quenched samples were then harvested by centrifugation, resuspended in 1 mL of 10 mM sodium azide solution, transferred to a 1.5 mL tube, harvested again, supernatant was removed, pellets were frozen at -80°C, and analyzed using the blotting procedure described below.

Western blotting. Frozen yeast pellets were thawed on ice, combined with 200 µL of ice-cold TCA buffer (10 mM Tris pH 8.0, 10% TCA, 25 mM NH_4OAc, 1 mM Na_2EDTA), resuspended by vortex mixing, and incubated on ice for 10 minutes. Precipitated proteins were harvested by centrifugation at 4°C, supernatant was removed, the pellets were resuspended in 100 µL of resuspension buffer (100 mM Tris, 3% SDS, pH 11.0), and heated at 95°C for 5 minutes. The resuspended protein samples were then centrifuged to remove cellular debris, and the clarified lysate was transferred to a clean 1.5 mL tube. The concentration of protein in each sample was quantified in a 96-well

plate using a *DC* Protein Assay kit (Bio-Rad, 500-0111). Samples were then resolved by SDS-PAGE (40 μg of lysate per lane in a 10% Bis-Tris acrylamide gel), transferred to a nitrocellulose membrane (Bio-Rad, 0.45 μM, 162-0115), and the membranes were blocked for 15 minutes in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% v/v Tween-20, pH 8.0) supplemented with 5% w/v dry milk.

Primary antibodies that recognize phosphorylated Fus3 and Kss1 (anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) mAb from Cell Signaling (4370S), 1:2000 dilution, overnight incubation), Fus3 (pAb from Santa Cruz (sc-6773), 1:500 dilution, 4 hour incubation), Gpa1 (pAb from our lab, 1:1000 dilution, 2-3 hour incubation), glucose-6-phosphate dehydrogenase (G6PDH load control) (pAb from Sigma-Aldrich (A9521), 1:50,000 dilution, 1 hour incubation), and phospho-AMPKα (mAb from Cell Signaling (4188), 1:2000 dilution, overnight incubation) were made fresh for each use in TBS-T supplemented with 10 mM sodium azide and 2.5% w/v bovine serum albumin. After incubation with primary antibody, membranes were washed with TBS-T, incubated for one hour with anti-rabbit (Bio-Rad, 170-5046) or anti-goat (Bio-Rad, 170-5047) secondary antibodies conjugated to horseradish peroxidase (in TBS-T with 1% w/v dry milk), washed again with TBS-T, and incubated with 1 mL of Western Lightning® Plus-ECL enhanced chemiluminescence substrate (Perkin Elmer, NEL105001EA) for 1 minute. Protein bands were visualized immediately by exposure to film, and quantified using the software ImageJ (National Institutes of Health, ver. 1.44o).