On the intracellular pH of baker’s yeast

Orij, P.J.

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CHAPTER 5

Phosphatidic acid is a pH biosensor that links membrane biogenesis to metabolism

Barry P. Young1*, John J. H. Shin1*, Rick Orij3, Jesse T. Chao1, Shu Chen Li1, Xue Li Guan4,5, Anthony Khong6, Eric Jan6, Markus R. Wenk4,7,8, William A. Prinz9, Gertien J. Smits3 & Christopher J. R. Loewen1,2

1) Department of Cellular and Physiological Sciences and 2) The Brain Research Centre, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada, 3) Department of Molecular Biology and Microbial Food Safety, University of Amsterdam, Amsterdam, 1018 WV, Netherlands, 4) Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, 5) Department of Biochemistry, University of Geneva, Sciences II, 30 Quai Ernest Ansermet, CH-1211 Geneva, Switzerland, 6) Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada, 7) Department of Biological Sciences National University of Singapore, Singapore, 8) Swiss Tropical and Public Health Institute, University of Basel, Socinstrasse 57, P.O. Box 4002, Basel, Switzerland, 9) Laboratory of Cell Biochemistry and Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

* Most of the experiments for this chapter were conducted by Barry Young and John Shin, who contributed equally to this work and share first authorship.

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Recognition of lipids by proteins is important for their targeting and activation in many signaling pathways, but the mechanisms that regulate such interactions are largely unknown. Here we found that binding of proteins to the ubiquitous signaling lipid phosphatidic acid (PA) depended on intracellular pH and the protonation state of its phosphate headgroup. In yeast, a rapid decrease in intracellular pH in response to glucose starvation regulated binding of PA to a transcription factor, Opi1, that coordinately repressed phospholipid metabolic genes. This enabled coupling of membrane biogenesis to nutrient availability.
Lipid signaling in yeast is regulated by pH.

The hydrophobic portions of lipids can be sensed by hydrophobic protein domains that are often membrane-inserted. Soluble protein domains recognize lipids by interacting predominately with their hydrophilic headgroups. Recruitment of proteins to membranes is dependent on the concentration of their target lipid in the bilayer. Membrane-associated transcription factors sense changes in the levels of key signaling lipids, enabling direct feedback regulation of lipid metabolism [1-3]. In yeast, phospholipid metabolism is regulated by the transcriptional repressor Opi1, part of a lipid-sensor complex in the endoplasm reticulum (ER) (Fig. 1) [3]. Opi1 is sequestered on the ER by binding both PA and the tail-anchored ER protein Scs2. Addition of inositol results in the rapid depletion of PA, release of Opi1 from the ER and translocation of Opi1 to the nucleus [3]. Nuclear Opi1 represses the Ino2/4 transcriptional activator complex, which binds a cis regulatory element, UAS_{INO}, found in many phospholipid metabolic genes [4].

Fig. 1. Regulation of lipid metabolism by the ER lipid-sensor. Scs2 and PA on the ER directly bind and sequester Opi1. Inositol regulates the level of PA in the ER through its metabolic conversion to phosphatidylinositol (PI). \textit{INO1} is the gene most highly regulated by inositol providing a negative feedback loop for transcriptional control of phospholipid metabolism. Addition of excess exogenous inositol causes a rapid depletion of ER PA and translocation of Opi1 to the nucleus. Once in the nucleus, Opi1 binds to the Ino2/4 transcriptional activator and represses UAS_{INO}-containing genes. This also involves recruitment of the Sin3 histone deacetylase complex (not shown).
Fig. 2. Results of the inositol auxotrophy screen. (A) Protein complexes and pathways that regulate lipid metabolism. Nodes represent genes identified as inositol auxotrophs in our screen and are coloured by pathway or complex. Edges indicate experimentally determined physical interactions. Only nodes with a minimum of one connection are drawn. (B) Enrichment of genes in our dataset grouped according to gene ontology. Fold enrichment is defined as the frequency of a given category in our dataset relative to the frequency of that category in the whole genome.
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Of the genes regulated by inositol and Opi1, INO1 is the most highly regulated [4]. INO1 encodes the rate-limiting enzyme in inositol biosynthesis; thus inositol auxotrophy is a sensitive measure of expression of the INO1 gene and the status of the ER lipid-sensor. We screened the haploid yeast deletion collection for sensitivity to growth in the absence of inositol. Inositol auxotrophy in mutant yeast strains can be due to the translocation of Opi1 from the ER to the nucleus. This leads to repression of transcription of a number of genes including INO1, resulting in impaired growth on medium lacking inositol. Therefore, by comparing the growth of yeast deletion strains on medium with and without inositol, a set of mutants was identified that potentially have mislocalized Opi1.

To confirm that Opi1 translocation was indeed responsible for the inositol auxotrophy, the screen was repeated with the OPI1 gene knocked-out in all deletion mutant strains. Those strains whose inositol auxotrophy was due to Opi1-induced repression of INO1 were able to grow on medium lacking inositol since Opi1 was no longer present to repress INO1 transcription. 231 mutants were identified with significant growth defects (Fig. 2). Most of these were rescued by deletion of Opi1 (Fig. 3A). The ∆ino1, ∆ino2 and ∆ino4 mutants, which act downstream of the ER lipid-sensor, were not rescued (Fig. 3B). The ∆scs2 mutant was rescued as expected. Genes that govern intracellular pH (pHi) were enriched in our dataset (Fig. 2), including all fourteen subunits of the vacuolar ATPase (V-ATPase) complex and the four factors in the ER responsible for its assembly [5] (Fig. 3C). The V-ATPase governs pH in part through regulation of Pma1 [6], a P-type H⁺ ATPase of the plasma membrane (PM) that is the master regulator of pHi [7]. A hypomorphic allele of PMA1 (pma1-007) that results in a 50% reduction in expression and activity of the Pma1 protein [8] was also an auxotroph (Fig. 3D). TRK1, a K⁺ transporter of the PM that activates Pma1 [6] was also identified.

In addition to the pH-related genes, other components and functions were also enriched, including transcription, chromosome organization and biogenesis, protein kinase activity and glycoprotein biosynthesis, some of which have been described previously [9, 10] (Fig. 2).

As we had no foresight that a role for pH would be discovered, the medium used in our screen was unbuffered. However, we have measured the pH of this
type of medium and found that it was maximally pH 4.3. This will become further acidified with the growth of yeast and therefore it is not possible to accurately predict the local pH for each mutant colony in the screen. It is likely that the acidification of the unbuffered medium enabled the identification of the pma1-007 and ∆trk1 mutants as these are not auxotrophic in medium buffered at pH 5.

Fig. 3. Genome-wide screen for regulators of phospholipid metabolism. (A) Inositol auxotrophy of ~4800 deletion mutants and effect of deletion of OPI1. Plotted are log₂ values of ratios of colony sizes for growth of mutants in the absence vs presence of inositol. Single mutants are plotted on the x-axis and double mutants with ∆opi1 on the y-axis. (B) Inositol auxotrophy of known regulators of phospholipid metabolism and rescue by ∆opi1. Plotted are ratios of colony sizes for growth of mutants in the absence vs presence of inositol (-Ino/+Ino). (C) Inositol auxotrophy of V-ATPase deletion mutants. Mutants are grouped by V-ATPase domain (V₁ - peripherally associated subunits, V₀ - membrane associated subunits) or factors required for V-ATPase assembly. Genes in parentheses indicate deletion of an overlapping dubious ORF and may not be true nulls. (D) Inositol auxotrophy of pma1-007 and ∆trk1 mutants. Error bars indicate SD.
Because the *pma1-007* and *Δtrk1* mutants have an impaired capacity to pump protons out of the cell, they should be sensitive to acidification of the cytosol. To test this we subjected the strains to acid-stress by growing them on medium buffered at low pH. Unlike WT, pH$_i$ in the *pma1-007* and *Δtrk1* mutants decreased with acidification of the medium (Fig. 4A). To test whether cytosolic acidification causes derepression of Opi1, we measured transcription of Opi1-dependent genes by reporter assay [3] at pH 3, 4 and 5. We found almost complete repression in the *pma1-007* mutant at lowered pH, which was alleviated by deletion of *OPI1* (Fig. 4B and Fig. 5). WT showed a modest decrease in UAS$_{INO}$ expression that was also dependent on *OPI1*. As expected, derepression of Opi1 resulted in inositol auxotrophy of *pma1-007* and *Δtrk1* cells at low pH (Fig. 4C). This was in contrast to the *Δscs2* mutant, which remained an inositol auxotroph at all pH values. Deletion of *OPI1* in the *pma1-007* mutant rescued its inositol auxotrophy at each pH. GFP-Opi1 accumulated in the nucleus of *pma1-007* cells, particularly at lowered pH (Fig. 4D), consistent with the decrease in UAS$_{INO}$ expression. The drug ebselen, found to inhibit Pma1 in vitro [11], caused an immediate drop in pH$_i$ of both WT and *pma1-007* cells to ~ 6.3 (Fig. 4E). Whilst WT cells stabilized at pH$_i$ ~ 6.4, pH$_i$ of *pma1-007* cells continued to decrease indicating that the mutant is more sensitive, likely as a result of reduced gene dosage [11, 12]. Within 5 min, ebselen caused GFP-Opi1 to translocate from the ER to the cytosol and nucleus (Fig. 4F). Thus, pH$_i$ regulates the localization and function of Opi1 and is a signal regulating transcription of phospholipid metabolic genes.
Fig. 4. pH regulates phospholipid metabolism. (A) pH of mutants grown in medium at pH 3, 4 and 5 compared to WT (*, vs WT at given pH, p < 0.001). (B) UAS_{INO} reporter expression measured in different mutants grown at pH 3, 4 and 5 (*, vs pH 5, p < 0.001). (C) Growth of mutants in the absence of inositol at varying pH at 37 °C. (D) Nuclear localization of GFP-Opi1 in cells grown at pH 3 and 5 quantified by confocal microscopy (*, vs WT at given pH, p < 0.005; **, vs pma1-007 at pH 5, p < 0.01). (E) Effect on pH after addition of 100 µM ebselen to WT and pma1-007 cells grown in medium at pH 5 (*, vs WT at given time point, p < 0.05). (F) Effect on the localization of GFP-Opi1 5 min after addition of 100 µM ebselen (ebs). Arrows indicate ER localizations (closed - cortical, open - nuclear envelope); arrowheads indicate cytoplasmic (closed) and nuclear (open) localizations. Error bars indicate SEM in A, D and E and SD in B. The scale bar indicates 2 µm.

Fig. 5. UAS_{INO} Reporter Assays. Activities for the different mutants were measured at the given pH in the presence of 1 mM inositol. UAS_{INO} expression in both WT and the pma1-007 mutant was repressed by addition of inositol compared to in the absence of inositol, except for pma1-007 at pH 3 (*), indicating that UAS_{INO} expression at pH 3 in the absence of inositol is fully repressed. Deletion of OPI1 prevented inositol-mediated repression.

We asked whether pH affected the binding of Opi1 to PA. A basic domain in Opi1, Q2, directly binds the predominant pool of yeast PA, located in the PM [3]. Ebselen caused GFP-Q2 to delocalize from the PM (Fig. 6A). This was also true for Spo20 (Fig. 6B), the other verified PA-binding protein in yeast [13]. PM delocalization with ebselen was not due to endocytosis (fig. 7A). To control pH precisely, we treated yeast with the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) over a range of pH [14] and quantified GFP-Q2 localization to the PM. GFP-Q2 delocalized as pH decreased from 7.2 to 6.4 (Fig. 6, C and D). In contrast, localization of a probe for phosphatidylserine in the PM, GFP-Lact-C2 [15], did not change (fig. 7B). Thus, the binding of proteins to PA in vivo is sensitive to pH.
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**Fig. 6.** pH governs the binding of Opi1 to PA through its protonation state. (A) Localization of GFP-Q2 after 5 min ebselen treatment. (B) Localization of the PA-binding domain of Spo20 (GFP-Spo2061-91) after 5 min ebselen treatment. (C) Treatment of yeast expressing GFP-Q2 with CCCP buffered at the indicated pH. (D) Quantification of PM localization of GFP-Q2 with CCCP treatment (*, vs pH 6.4; **, vs pH 6.8; ***, vs pH 7; p < 0.005). (E) GFP-Q2 localization in Δvma2 cells. (F) pH measured in WT and Δvma2 cells grown in pH 5 medium (*, p < 0.0001). (G) Total PA measured by mass spectrometry in WT and Δvma2 cells grown in pH 5 medium (*, p < 0.0001). (H) Binding of Q2 and Q2\textsuperscript{C3M} to liposomes containing 10 mol% PA / 40 mol% phosphatidylethanolamine (PE) over a range of pH (*, vs pH 6.4; **, vs pH 6.8; ***, vs pH 7.2; p < 0.05). (I) Binding of Q2 to liposomes (0, 100, 200 µM total lipid) containing 50 mol% PA or methyl-PA at pH 7.2. (J) Binding of Q2 and Q2\textsuperscript{C3M} to liposomes containing 20 mol% methyl-PA / 40 mol% PE over a range of pH. Error bars indicate SD except in D (SEM). The scale bars indicate 2 µm.
GFP-Q2 was delocalized in a V-ATPase mutant, Δvma2, that had an acidified cytosol (Fig. 6, E and F). GFP-Lact-C2 localization was unaffected in this mutant (Fig. 7C). Consistent with an inability of Opi1 to bind PA in Δvma2 cells, GFP-Opi1 was translocated to the nucleus in this mutant, which also had decreased UASINO expression and was an inositol auxotroph (Fig. 8). These phenotypes were not due to decreased PA levels, which were instead elevated ~ 70% in the mutant (Fig. 6G). Thus, Opi1 failed to bind PA at lowered pH in this mutant.

The Δvma2 mutant was one of the strongest auxotrophs identified in our screen and was rescued by deletion of OPI1 indicating that its inositol phenotype was solely due to derepression of Opi1. Its inositol auxotrophy was verified by spot assay (Fig. 8C) and we found a 50% reduction in UASINO expression (Fig. 8B). Consistent with phospholipid metabolic genes being significantly downregulated in this mutant, GFP-Opi1 was also largely translocated to the nucleus (Fig. 8A). GFP-Q2 failed to localize to the PM (Fig. 6E), which suggested that dysregulation of Opi1 in this mutant was a result of its inability to bind PA.
Lastly, we found Δvma2 cells had pH_i ~ 6.6 (Fig. 6F), which was below the critical threshold we determined for Opi1 binding PA both in vivo (Fig. 6D) and in vitro (Fig. 6H). We chose to measure PA in this mutant by mass spectrometry because of the high accuracy of this method. However, since it is less sensitive than other methods, and PA is a scarce lipid, it was necessary to measure total PA. We feel this was justified given that GFP-Q2 failed to localize to the PM, which is the location of the predominant pool of PA in yeast [3]. Therefore, the result that Δvma2 cells had nearly twice as much PA as WT indicates that lowered pH_i in this mutant prevents Opi1 from binding PA and supports our conclusion that Opi1 preferentially binds to deprotonated PA. Nevertheless, it cannot be completely ruled-out that the change in pH_i affects the PA binding site in Opi1. However, this interpretation is contrary to our in vitro liposome binding data with methyl-PA (Fig. 6J) and also the predictions made by the electrostatic/hydrogen bond switch mechanism for PA-protein interactions [16].

Fig. 8. Phenotypes of Δvma2 cells. (A) Nuclear translocation of GFP-Opi1 in Δvma2 cells compared to WT grown in pH 5 medium (*, p < 10^{-11}). (B) UAS{ino} expression of WT and Δvma2 cells grown in pH 5 medium (*, vs WT - inositol, p < 10^{-8}). (C) Inositol auxotrophy of Δvma2 cells grown in pH 5 medium. Error bars indicate SEM in A and SD in B.

Next we bound recombinant Q2 to liposomes containing PA at varying pH. We found a near linear increase in binding between pH 6.4 and 7.6 (Fig. 6H and Fig. 9D). Three basic amino acids in Q2, 136K137K138R, are thought to participate in electrostatic interactions with the negatively charged headgroup of PA [3]. Binding of the triple alanine substitution mutant (Q2^{C3M}) [3] to PA between pH
6.4 and 7.6 no longer depended on pH (Fig. 6H and Fig. 9D). Thus, the direct interaction between Q2 and PA is sensitive to pH.

The structures of PA and methyl-PA are shown for comparison in Fig. 9A. We included 40 mol% PE in liposomes as it enhanced specific binding of Q2 to lower, near physiological levels of PA (Fig. 9B), as has been shown for Raf kinase [16]. PE is a cone-shaped lipid similar to PA and is thought to increase the accessibility of PA to the protein [16]. This level of PE also better approximates the composition of the PM and ER [17]. That binding of Q2 was also enhanced by PE (Fig. 9B) indicates this binding was specific and relevant. This was also true for binding to methyl-PA (Fig. 9C). In the pH titration experiments (Fig. 9D) we used methyl-PA at 20 mol% to detect specific binding of Q2 above background because of the lower affinity of Q2 for this lipid (Fig. 9, B and C). It is important to note that PE only had an effect on binding in the presence of PA or methyl-PA.

Fig. 9. Reconstitution of pH-dependent binding to PA in vitro. (A) Structure of PA and methyl-PA at physiological pH. Dashed lines indicate shared hydrogen bonds. (B) Binding of Q2 and Q2\textsuperscript{C3M} to PA is specific and increases with increasing PE. Binding was performed at pH 7.2 with 2.4 mM liposomes containing 20 mol% PA and the indicated amount of PE. “No PA” indicates liposomes containing 40 mol% PE but no PA. (C) PE enhances specific binding to methyl-PA. Binding was performed at pH 7.2 with 2.4 mM liposomes containing 20 mol% methyl-PA and the indicated
amount of PE. “No m-PA” indicates liposomes containing 40 mol% PE but no methyl-PA. Note the weaker binding to methyl-PA than to PA. (D) Representative gels of binding at varying pH. Binding was performed with 2.4 mM liposomes containing 40 mol% PE and either 10 mol% PA or 20 mol% methyl-PA at the indicated pH.

Unlike other phospholipids, the phosphate headgroup of PA is a monoester and has a second pK\textsubscript{a} measured in model membranes to be between 6.6 and 7.9, depending on their phospholipid and protein composition [18, 19]. Changes in the ionization state of PA might thus be responsible for the observed pH-dependent binding of Opi1. We tested binding of Q2 to methyl-PA, which bears a methyl-group substitution on the phosphate and lacks the second pK\textsubscript{a} (fig. 9A). Binding remained specific (fig. 9C), but was considerably weaker (Fig. 6I) and was pH-independent (Fig. 6J and Fig. 9D). Thus, Opi1 had higher affinity for deprotonated PA, consistent with the proposed electrostatic/hydrogen bond switch mechanism for the interaction of proteins with PA [16].

We identified several components of the glucose response pathway in our screen (Fig. 10A). These included the yeast homologue of the AMP-activated kinase, Snf1, and its regulatory subunit Snf4 [20]. We also identified Bmh1, which is a conserved 14-3-3 protein that has been found to bind Snf1 and Snf4 in high throughput studies [21, 22] and plays a role in maintaining glucose repression [23]. Bmh1 may also regulate Pma1 [24], thus providing a possible link between Snf1 and regulation of pH. This is supported by our data that the Δsnf1 and Δsnf4 mutants were pH-dependent auxotrophs (Fig. 10B). We also identified Grr1, an Fbox protein that is a major regulator of glucose signaling [20], which is also regulated by Bmh1 [25].
Fig. 10. Glucose signaling genes identified in the inositol auxotrophy screen. (A) Results from screen. All mutants were rescued by deletion of OPI1 except Δgrr1 which was not done. (B) Inositol auxotrophy assays at varying pH show that the AMP-dependent kinase mutants Δsnf1 and Δsnf4 are pH-dependent auxotrophs. Note the stronger phenotypes at pH3 that are rescued by addition of inositol.

Because glucose-starved yeast exhibit a rapid drop in pH to ~ 6 [6] and our screen identified several major regulators of glucose signaling (Fig. 10), we hypothesized that glucose might be a physiological pH signal. Glucose starvation resulted in translocation of GFP-Opi1 from the ER to the nucleus (Fig. 11, A and B), which correlated with the drop in pH (Fig. 11C). INO1 transcription was also repressed on a similar timescale (Fig. 11D). Translocation of GFP-Opi1 was not dependent on known modulators of PA levels (Fig. 12), suggesting glucose acted independently of changes in the concentration of PA. Consistently, PA levels measured in ER microsomes isolated after 20 min of glucose starvation did not change significantly.

We tested whether the glucose starvation signal is separate from that generated by addition of inositol. The enzyme phosphatidylinositol synthase, encoded by the PIS1 gene, is responsible for the incorporation of inositol into PI, which results in the rapid depletion of ER PA and translocation of Opi1 to the nucleus [3]. We performed a glucose starvation experiment in a pis1 mutant that prevents inositol-mediated translocation of Opi1 [3]. We found that translocation in the mutant occurred at the same rate as in WT (Fig. 12B), indicating that the glucose starvation signal was not mediated by conversion of PA into PI. This was also supported by our phospholipid analysis of ER microsomes that showed no increase in the level of PI upon glucose starvation.

We also tested for roles for enzymes known to directly affect PA levels. These were: PA phosphatase (Pah1), which hydrolyzes PA to diacylglycerol [26]; phospholipase D (Spo14), which produces PA by hydrolysis of PC [27]; and diacylglycerol kinase (Dgk1), which phosphorylates diacylglycerol to make PA [28]. Deletion of the genes encoding these enzymes did not affect the localization of GFP-Opi1 to the ER or its translocation in response to glucose starvation (Fig. 12, C-E). We did not identify SPO14 or DGK1 in our inositol auxotrophy screen indicating that they were not required to maintain the pool of
PA in the ER detected by Op1. Δpah1 cells have elevated PA and derepressed INO1 transcription indicating that Op1 is hyper-repressed [28]. That GFP-Op1 still translocated upon glucose starvation in Δpah1 cells was additional evidence that the glucose signal was independent of a decrease in the level of ER PA.

Reg1 is the glucose-signaling-specific regulatory subunit for Glc7, yeast’s protein phosphatase type 1. Glc7 is implicated in repression of Pma1 [29] and Δreg1 yeast fail to repress INO1 and overproduce inositol [30], suggesting that...
Reg1/Glc7 regulates pH$_i$ through Pma1. Deletion of REG1 rescued the inositol auxotrophy of the pma1-007 mutant (Fig. 11E). Pma1 activity in the ∆reg1 mutant was higher and failed to repress upon glucose starvation (Fig. 11F). Deletion of REG1 attenuated the rapid drop in pH$_i$ in glucose starved cells (Fig. 11C). Thus, Reg1 repressed Pma1 in response to glucose starvation. GFP-Opi1 translocation was delayed in ∆reg1 cells (Fig. 11B and Fig. 12). In both WT and ∆reg1, translocation coincided with a drop in pH$_i$ below 6.9, consistent with the reduced affinity of Opi1 for protonated PA.

Fig. 12. GFP-Opi1 translocation upon glucose starvation in various mutants affecting PA. (A) Schematic of pathways that regulate PA levels. Genes encoding various enzymes are shown in italics. PC - phosphatidylcholine, DAG - diacylglycerol, PI - phosphatidylinositol. (B) Kinetics of GFP-Opi1 translocation are unaltered in the pist1-234 mutant, which prevents translocation in response to inositol. Note the absence of cortical ER-localized GFP-Opi1 at 5 min (arrows indicate localization to the cortical ER). (C-E) Translocation is unaltered in mutants of known regulators of PA. Note the absence of cortical ER-localized GFP-Opi1 after glucose starvation. Pah1 - PA hydrolase; Spo14 - phospholipase D; Dgk1 - DAG kinase; -Dex, 5 min after glucose starvation. (F) GFP-Opi1 translocation is delayed in ∆reg1 cells. Time after glucose starvation is shown. Note the
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presence of cortical ER-localized GFP-Opi1 at 5 and 10 min. Open arrows - nuclear envelope; closed arrows - cortical ER.

Phosphatidic acid signaling can be dynamically regulated by changes in pH (Fig. 13). This involves a change in the protonation state of the phosphate headgroup, making the lipid a pH biosensor. A pH-sensing role for lipids may not be limited to PA because phosphatidylinositol phosphates and ceramide-1-phosphate have pKa values within the physiological range [31, 32]. Given the established roles for these lipids in signaling and the universality of pH regulation in biology, pH-dependent protein-lipid interactions may be important in a wide variety of systems.
Fig. 13. pH-dependent interaction of proteins with PA. (A) Membrane biogenesis is coupled to metabolism through pH-dependent lipid signaling (PAH, protonated PA; PA−, deprotonated PA). (B) PA-binding proteins have higher affinity for deprotonated di-anionic PA. Based on the electrostatic/hydrogen bond switch model [16], this occurs as a result of hydrogen bonding between the primary amines of lysines and arginines in the protein and the phosphate headgroup of PA, and through electrostatic interactions between these groups. Our data with Q2C3M demonstrate the requirement of lysines and arginines for discriminating between protonation states of PA.

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METHODS

Yeast growth conditions. For microscopy, yeast strains were grown at 30 °C to early log phase in synthetic complete medium without inositol (MP Biomedicals) or supplemented with 1 mM inositol if stated. For inositol auxotrophy assays, yeast were grown at 37 °C for 36 h. Ebselen was used at a final concentration of 100 µM with 3x10⁷ cells/ml for 5 min unless otherwise stated. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) treatment was done as previously described [14]; briefly cells were grown in the presence of inositol to log phase and then resuspended in 25 mM HEPES, pH 6.2 - 7.4, 200 mM KCl, 1 mM CaCl2, 2% glucose, 0.5 mM CCCP, at 30 °C. After a 10 min incubation, confocal images of cells were taken. When indicated, medium pH was buffered with 50 mM sodium phosphate, 50 mM sodium succinate. For glucose-starvation experiments, strains were grown in medium containing 2% glucose, collected by centrifugation, washed once, and resuspended in the same medium lacking glucose.
Construction of double mutants and GFP-tagged strains. *OPI1* was deleted or tagged with GFP in each strain of the yeast deletion collection using Synthetic Genetic Array (SGA) techniques as described previously [33]. To delete *OPI1*, each strain in the deletion collection was crossed with strain Y7092 [33] knocked out at the *OPI1* locus with a cassette conferring resistance to nourseothricin. To GFP-tag *OPI1*, each strain was crossed with Y7043 [33] containing a HIS3-GFP cassette integrated at the 5' end of the *OPI1* open reading frame. The constitutive portion of the PHO5 promoter was used as the promoter. For ER microsome isolation, a WT BY4741 strain was constructed that contained endogenous Erg11 and Acp1 tagged at their C-termini with GFP by PCR knock-in.

Plasmids. The plasmids expressing P_{ACT}-pHluorin and the GFP-Q2 fusion have been described previously [3, 14]. pACT1-pHluorin is restricted to non-commercial parties. GFP-Spo20 plasmid [13] was a kind gift of A. Neiman. The GFP-Lact-C2 plasmid [15] was a kind gift of S. Grinstein. The Psr1^1-28^GFP plasmid contains the first 28 amino acids of Psr1, which is palmitoylated (a kind gift of T. Levine). MBP-Q2 plasmids were as previously described [3].

Yeast robotics and image acquisition. Yeast arrays were manipulated using a RoToR HDA robot (Singer Instruments) at a density of 1536 spots per plate. Images of plates were acquired using a flatbed scanner. To identify inositol-sensitive strains, mutant arrays were copied to synthetic complete medium lacking or containing 1 mM inositol, then copied a second time, making three copies on the same medium. Plates were incubated at 37 °C. After acquiring images of plates, the area of each spot was measured using ColonyImager software [33]. ColonyScorer software [33] was used to normalize to the median spot size on each plate, correct for positional effects and derive means and standard deviations for each spot.

Analysis of growth rates. Following data acquisition, sensitivity to lack of inositol was defined as the ratio of the relative growth of a given spot on experimental medium (minus inositol) to that on control medium (plus inositol). This ratio was calculated for each spot in the array with replica measurements assumed to follow a Gaussian distribution. Strains were identified as sensitive if the ratio for that strain was more than 95% certain to be greater than 3 standard deviations away from the background variance of the deletion collection (s.d. = 0.053). This corresponds to a probability of greater than 0.95 that the ratio is less than 0.84. Strains were defined as sick if the growth rate in the presence of inositol was less than 20% of the median growth of strains in the deletion collection; these strains were discarded from further analysis.

To identify rescue of inositol-sensitivity by deletion of *OPI1*, the inositol-sensitivity was calculated for each double mutant strain. If the strain was no longer classified as sensitive using the criteria described above, then it was deemed to be fully rescued. If the strain was still deemed to be inositol sensitive, then a two-tailed t-test was performed based on the difference between the sensitivity of the double mutant and the sensitivity of the single mutant. The strains were determined to have been partially rescued if the associated p-value was < 0.005. If strains grew at less than 20% of the median growth rate of the deletion collection or not significantly better than the corresponding *OPI1* strain in the absence of inositol, they were classified as not rescued. It was not possible to generate double mutants with ∆opi1 and approximately 18% of the inositol-sensitive deletion mutants; in
nearly all cases this was because the single deletion mutant strains were not viable on the growth medium used to germinate haploid cells in the SGA procedure.

Bioinformatic analysis. Enrichment of data sets for Gene Ontology terms was performed using the Gene Ontology Term Finder at the Saccharomyces Genome Database (http://db.yeastgenome.org/cgi-bin/GO/goTermFinder.pl). Interaction networks were visualized with Osprey (6) using data from physical interaction databases.

Measurement of pHi. For pHi determinations, strains expressing PACT-pHluorin were cultured at 30 °C in low fluorescence medium (synthetic defined medium without riboflavin or folic acid, 1 mM inositol, 2% glucose), buffered with 25 mM citrate to pH 5.0 in CELLSTAR black polystyrene clear-bottomed 96-well microtiter plates. At mid-log phase, fluorescence emission was determined in a SpectraMax Gemini XS spectrofluorometer (Molecular Devices) at 512 nm after excitation at 390 nm and 470 nm. Both intensities were corrected for the background fluorescence of a control strain (empty vector) subjected to identical experimental conditions. The ratio of the two intensities was related to that of a calibration curve of cells permeabilized with digitonin (100 µg/ml for 10 min) and resuspended in buffers with pH between 5.0 and 9.0. Each experiment represents the average and standard deviation of four independent transformants assayed twice.

Assay for β-galactosidase activity. Yeast strains expressing the UASiNO reporter plasmid pTL5ILZ [3] were grown at 30 °C in synthetic complete medium without inositol, buffered at pH 5, to log phase. For Fig. 2B & S3, these strains were resuspended in synthetic complete medium with and without inositol, buffered to pH 3, 4, or 5 at an OD600 of 0.2. After incubation at 30 °C for 4 hours, cultures were assayed for β-galactosidase activity as previously described [3]. For Fig. S6B, strains were resuspended in the same pH 5 medium at a starting OD600 of ~ 0.03 and incubated at 30 °C until an OD600 of 0.8 was reached, whereupon β-galactosidase activity was assayed. All assays were performed with a minimum of three replicates.

Northern blot analysis. RNA was isolated from wild type cells grown in the absence of inositol by SDS/acid phenol extraction [34] at various time points during glucose starvation in synthetic complete medium lacking glucose. Equal amounts of RNA were analyzed on Northern blots, probing for INO1 mRNA. Following quantitation using a phosphorimager, signals were normalized to the first time-point.

Pma1 ATPase activity assays. Plasma membrane fractions were obtained as described previously [6] for cells grown in the presence of glucose, or glucose-starved for 20 min. The vanadate-sensitive ATPase activity of membrane preparations was determined as before [6]. All assays were performed using three replicates.

Phospholipid analysis by mass spectrometry. Membranes from log phase yeast grown in medium in the absence of inositol at pH 5 were homogenized by liquid nitrogen grinding. The cell lysate was cleared of debris by sedimentation at 1500 g and the supernatant was sedimented at 40,000 g for 30 min. The 40,000 g pellet was used for subsequent lipid analysis. Lipids were extracted and analyzed as previously described [35]. Separation and quantitation of lipids was
performed using normal phase LC-MS with an Agilent 1200 HPLC system and a 3200 Q-Trap mass spectrometer (Applied Biosystems). Lipid levels were quantified using spiked internal standards (Avanti Polar Lipids) [35]. Phosphatidic acid level is expressed as mol% of total phospholipids measured.

**Isolation of ER microsomes and measurement of glycerophospholipids by HPLC.** A yeast strain was constructed with the genes *ACP1* and *ERG11* tagged with GFP to enable identification of ER microsomes and assess contamination during subcellular fractionation. Acp1 localizes to mitochondria and Erg11 to the ER. Yeast cells expressing Acp1-GFP and Erg11-GFP were grown to log phase in synthetic complete medium lacking inositol and subjected to subcellular fractionation as previously described [36]. Glucose starvation was performed for 20 min. After spheroplasting and homogenization, mitochondria were pelleted at 12,000 *g* in a Beckman JA-25.5 rotor. The post-mitochondrial supernatant was then sequentially pelleted at 30,000 *g* and 40,000 *g* to obtain ER microsomes. Both the 30,000 *g* and 40,000 *g* pellets were tested for mitochondrial contamination and enrichment for ER by Western blot analysis with anti-GFP antibodies. The 40,000 *g* pellet was checked for plasma membrane contamination by assaying for Pma1 activity. No detectable Pma1 specific activity was found. The 40,000 *g* microsomal membrane fraction was subjected to lipid analysis by HPLC (see below).

Lipids were extracted as described [37], dried under N₂, resuspended in CHCl₃, injected onto a LiChrospher 100 Diol 250 x 4.6 mm (5 µm) column (Agilent Technologies) that was heated to 65 °C, and separated basically as described [38]. Solution A was hexane:2-propanol:1-butanol:tetrahydrofuran:isoctane:water (64.5:17.4:7:5:5:1) and solution B was 2-propanol:1-butanol:tetrahydrofuran:isoctane:water (73:7:5:5:10). Ammonium acetate was added to both solutions at 180 mg/L. Solution A was initially set at 100%. Solution B was then increased to 13% by 5 minutes, held at 13% until minute 18, and then increased 45% B by 42 minutes. The column was re-equilibrated with solution A for 10 min before each injection. Lipids were detected with a PL-ELS 2100 evaporative light scattering detector (Varian) with an evaporator temperature of 90°C, nebulizer temperature of 65°C, and a gas (N₂) flow rate of 1.8 l/min. The peaks containing the six major glycerophospholipids were identified by comparison to known standards (Avanti Polar Lipids).

**Liposome binding assays.** Binding of maltose binding protein-tagged Opi1 fragments to synthetic lipid vesicles was performed as described previously [3]. Briefly, binding reactions were carried out in 250 µl of binding buffer (BB; 125 mM KCl, 25 mM HEPES, 1 mM DTT, 25 µM CaCl₂, 1 mM AEBSF, 1 mg/ml soybean trypsin inhibitor) at pH 7.2 unless otherwise stated and incubated for 30 min at 22 °C before sedimentation at 50,000 rpm for 5 min (TLA120.2, Beckman). Liposome pellets were rinsed once with BB and resuspended in sample buffer for SDS-PAGE. For pH titration experiments, liposomes were prepared and rinsed in buffer at the same pH as used in the binding reaction. Liposomes contained 10 or 20 mol% dioleoyl PA or 20 mol% dioleoyl methyl-PA, 40 mol% dioleoyl phosphatidylethanolamine (PE), with the remainder consisting of dioleoyl phosphatidylcholine (PC), except for liposomes with 50 mol% PA or methyl-PA, which contained 50 mol% PC and no PE. All lipids were from Avanti Polar Lipids. To quantify the amount of protein bound, SDS-PAGE gels were stained with Coomassie brilliant blue and analyzed by densitometry using ImageJ [39]. To control for differences in the maximal binding capacity of different liposome
preparations, individual band densities were normalized to the mean density of all experimental bands for that gel. pH titration experiments were done in triplicate with three separate preparations of liposomes.

**Quantification of Confocal Microscopy Images.** Images were captured on a Pascal Laser Scanning Microscope (Zeiss). All confocal microscopy images were taken with identical microscope settings to enable direct comparison and quantification between images and samples. A minimum of 30 cells per time point was analyzed and every cell in each confocal field was counted. Nuclear localization of GFP-Opi1 was quantified by determining the mean GFP-Opi1 pixel intensity in the nucleus from a single confocal plane per cell. Mean pixel intensity for each cell was determined using ImageJ software [39] by drawing a line across the nucleus and plotting the pixel intensity using a 5 pixel simple moving average. The mean pixel intensity was then determined from this plot. GFP-Q2 and GFP-Lact-C2 localization to the plasma membrane and cytoplasm was quantified in the same way as above, except that the line drawn using ImageJ was across the diameter of the whole cell. The ratio of the peak pixel intensity at the perimeter of the cell to the mean pixel intensity of the cytoplasm for each cell was calculated.

**REFERENCES**

Lipid signaling in yeast is regulated by pH.


