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SPHINGOLIPIDS FUNCTION AS DOWNSTREAM EFFECTORS OF A FUNGAL PAQR RECEPTOR

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Running title: Sphingolipid-dependent signaling by yeast PAQR receptors

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Nonstandard abbreviations:

PAQR: Progestin and AdipoQ Receptor

IZH: Implicated in Zinc Homeostasis

D-*erythro*-MAPP: D-(2-myristoylamino)-3-phenylpropanol

FET: Ferric iron transporter

PHS: phytosphingosine

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Abstract

The Izh2p protein from *Saccharomyces cerevisiae* belongs to the newly characterized PAQR superfamily of receptors whose mechanism of signal transduction is still unknown. Izh2p functions as a receptor for the plant PR-5 defensin osmotin and has pleiotropic effects cellular biochemistry. One example of this pleiotropy is the Izh2p-dependent repression of *FET3*, a gene involved in iron-uptake. While the physiological purpose of *FET3* repression by Izh2p is a matter of speculation, it provides a reporter with which to probe the mechanism of signal transduction by this novel class of receptor. Receptors in the PAQR family share sequence similarity with enzymes involved in ceramide metabolism, which led to the hypothesis that sphingolipids are involved in Izh2p-dependent signaling. In this study, we demonstrate that drugs affecting sphingolipid metabolism, such as D-*erythro*-MAPP and myriocin, inhibit the effect of Izh2p on *FET3*. We also show that Izh2p causes an increase in steady state levels of sphingoid base. Moreover, we show that Izh2p-independent increases in sphingoid bases recapitulate the effect of Izh2p on *FET3*. Finally, our data indicate that the Pkh1p and Pkh2p sphingoid-base sensing kinases are essential components of the Izh2p-dependent signaling pathway. In conclusion, our data indicate that Izh2p produces sphingoid bases and that these bioactive lipids likely function as the second messenger responsible for the effect of Izh2p on *FET3*.

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The PAQR superfamily of proteins is comprised of membrane bound receptors (Tang et al., 2005) and includes two mammalian proteins that function as receptors for the hormone adiponectin, which represents a molecular link between obesity and type II diabetes. Other members of this family sense and respond to progesterone, making them excellent candidates for the long-sought-after receptors that mediate many of the rapid non-transcriptional effects of progesterone. Due to arguable similarity to G-protein coupled receptors, it has been proposed that PAQRs function as a new class of GPCR (Thomas et al., 2007). However, this assignment is highly debatable and, in reality, almost nothing is known about how these proteins transduce signals. What is needed to explore this complicated question is a simple system with which to study the functionality of PAQR receptors.

We chose to use the model eukaryote, *Saccharomyces cerevisiae*, to study these receptors for several reasons. First, the *S. cerevisiae* genome encodes 4 proteins - Izh1p, Izh2p, Izh3p and Izh4p - in the PAQR superfamily of receptors (Lyons et al., 2004) and the study of yeast orthologs as models for human proteins has been a remarkably fruitful method for characterizing poorly understood protein families (Foury, 1997). Second, the plant protein osmotin has been identified as an agonist for Izh2p, (Narasimhan et al., 2005) allowing us to probe receptor activation in this organism. Third, the simplicity and tractability of *S. cerevisiae* as a genetic system make it ideal for mapping signal transduction pathways. Indeed, *S. cerevisiae* has already been successfully used to probe the mechanism of signal transduction by a variety of receptor types (McEwan, 2001).

In order to map a biochemical pathway, it is helpful to have a measurable phenotype associated with a functional receptor that can be exploited. While attempting to elucidate the physiological role of Izh2p, we discovered that Izh2p negatively regulates the expression of a gene involved in iron-uptake called *FET3* (Kupchak et al., 2007). The reason for Izh2p-dependent regulation of *FET3* is still poorly understood, however, it is not critical to the goals of this study to understand why Izh2p regulates *FET3*. What is important is that we can use the effect of Izh2p on *FET3* as a reporter for receptor activity and as a tool to map the pathway of signals emanating from this receptor.

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We already used this system to partially map the signal transduction pathway and determined that Izh2p overexpression represses *FET3* by regulating transcription factors that exert opposing effects on a regulatory element in the *FET3* promoter. In addition, we determined that cAMP-dependent protein kinase (PKA) was essential for these effects (Kupchak et al., 2007). Most importantly, however, we demonstrated that the PAQR-dependent signaling pathway in yeast does not involve G-proteins, indicating that PAQRs are not GPCRs (Smith et al., 2008). Hence, we have already demonstrated the efficacy of this system for testing models of signal transduction and identifying novel players in the pathway. We also demonstrated that human PAQRs, when expressed in yeast, repress *FET3* in response to activation by their respective agonists (Kupchak et al., 2007; Smith et al., 2008), thus demonstrating that the mechanism of signal transduction may be conserved in the PAQR family.

The question remains; if PAQRs are not GPCRs, then what is the chemical second messenger produced by these receptors? A thorough analysis of the sequence of PAQR receptors reveals significant similarity to a group of enzymes known as alkaline ceramidases. Ceramidases hydrolyze ceramides to generate free fatty acids and sphingoid bases, which are a class of bioactive aminolipids known to function as potent second messengers. Indeed, the ceramide-sphingoid base ratio is used as a rheostat in eukaryotic cells to regulate a variety of important physiological functions (Kobayashi and Nagiec, 2003). In this study, we demonstrate that Izh2p overexpression elicits the accumulation of sphingoid bases and that sphingoid bases are capable of independently activating the signaling pathway downstream of Izh2p. This accumulation requires *de novo* sphingoid base and ceramide biosynthesis. Moreover, we demonstrate that *FET3* repression by Izh2p can be prevented by D-*erythro*-MAPP, an alkaline ceramidase inhibitor. Finally, we demonstrate that Izh2p-dependent *FET3* repression requires a pair of sphingoid base-sensing kinases. Based on these findings, we propose a model in which Izh2p in particular, and perhaps PAQR receptors in general, generate sphingoid base second messengers.

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Experimental Procedures

Yeast strains, plasmids and materials. Unless specifically noted, all wild type and single mutants strains were purchased from Euroscarf (Frankfurt, Germany) and are in the BY4742 background (*MATα; lys2Δ; ura3Δ; his3Δ; leu2Δ*; kanMX4 deletion cassette). The *ypc1Δydc1Δ* double mutant (*MATα; ade2; his3; leu2; trp1; ura3; can1; ypc1::LEU2; ydc1::TRP1*) and the isogenic W303-1A wild type *(MATα; ade2; his3; leu2; trp1; ura3; can1*) background strains were a gift of Dr. Howard Riezman at the University Basel, Switzerland (Schorling et al., 2001). *FET3-lacZ* was used as a *FET3* promoter/*lacZ* reporter plasmid and was described previously (Kupchak et al., 2007). *IZH2* and *YPC1* were cloned into pRS316-*GAL1* via gap repair as previously described (Lyons et al., 2004). Primers for cloning the *YPC1* gene into pRS316-*GAL1* by gap repair are shown in **Supplemental Data 1**. Fumonisin B_1, myriocin, D-*erythro*-MAPP and N,N-dimethylsphingosine were purchased from Cayman Chemical (Ann Arbor, MI). Phytosphingosine, dihydrosphingosine and C_2-phytoceramide were purchased from Avanti Polar Lipids (Alabaster, AL). Stearylamine and thaumatin were purchased from Sigma-Aldrich. NBD-C_{12}-phytoceramide and NBD-dodecanoic acid were provided by Dr. Yusuf Hannun. In this manuscript, we discuss many yeast genes with which a reader who is not in the field may not be familiar. In order to make it easier to read this manuscript, we have included a brief description of these genes and their human homologs in **Supplemental Table 1**.

Growth conditions and biochemical assays. For β-galactosidase and ferroxidase assays, cells were pre-grown in synthetic defined medium with 2% glucose as the carbon source and re-inoculated into Low Iron Medium (LIM) containing galactose to induce expression of genes driven by the *GAL1* promoter. Iron-deficiency and -repletion were generated by adding $FeCl_3$ back to LIM at either 1 μM or 1 mM final concentrations, respectively. 2% galactose was used to fully induce *GAL1*-driven genes, while modest overexpression was achieved by adding 0.05% galactose and 1.95% raffinose to the medium. Cells were allowed to grow to mid-log phase in LIM and β-galactosidase or ferroxidase assays were performed as

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previously described (Kupchak et al., 2007). For individual experiments, each data point was done in triplicate and the error bars represent ±1 standard error of the mean (SE).

Fresh stocks of phytosphingosine (PHS), dihydrosphingosine (DHS), stearylamine, D-*erythro*-MAPP, N,N-dimethylsphingosine (N,N-DMS), fumonisin B_1 (FB_1) and myriocin were made by dissolution in ethanol (D-erythro-MAPP, myriocin, N,N-DMS) methanol (FB_1) or 1:1 $CHCl_3$:MeOH (PHS, DHS, stearlyamine). For all experiments equal volumes of the appropriate solvent were added to untreated cells to control for vehicle effects. For all cases except myriocin, the drug was added upon re-inoculation of cells into LIM. In the case of myriocin, cells were treated with the drug for 1 hr prior to β-galactosidase assay. EC_{50} values were obtained using the web-based BioDataFit software using sigmoidal curve-fitting (http://www.changbioscience.com/stat/ec 50.html).

Deletion of the multidrug resistance transporter, Yor1p, causes cells to be hypersensitive to the antifungal drug fumonisin B_1 (FB_1) due to the inability to export the toxic compound (Mao et al., 2000a). For FB_1 sensitivity experiments, *yor1*Δ cells were grown in synthetic defined medium containing 2% galactose. FB_1 (from DMSO stocks) was added to a final concentration of 100 μM. Cells not treated with FB_1 were treated with an equivalent volume of DMSO vehicle. For each experiment, the growth of three independent colonies of each strain was tested. Error bars show ±1 SE.

Sphingolipid extraction and analysis. Overnight cultures of yeast were inoculated into synthetic defined medium containing 2% galactose, allowed to grow to mid-log phase and harvested at OD_{600} = 0.8. Cells treated with myriocin, FB_1 (from DMSO stocks) or C_2-phytoceramide (from ethanol stock) were so-treated for 30 min prior to extraction. Untreated controls were exposed to an equal volume of either DMSO or ethanol. Sphingolipids were extracted using a modified Bligh-Dyer protocol (Bligh and Dyer, 1959). Cell pellets from 30 mL of culture were vortexed with 3 mL of $CHCl_3$:MeOH (1:2) to extract total lipids, after which 0.8 mL of water was added. The suspension was transferred to a screw cap glass tube and left at 4ºC overnight. Cellular debris was pelleted at 3000 rpm for 10 min. The supernatant was

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transferred to a new glass tube and vortexed with 2 mL $CHCl_3$:H_2O (1:1). After 30 min, the mixture was centrifuged for 5 min at 3000 rpm to obtain a clean phase separation. The organic phase was quantitatively transferred to a new glass vial and dried down using a nitrogen evaporator. Dried lipids were dissolved in 300 μL of $CHCl_3$, of which 100 μL was transferred to another tube for phosphate determination. 0.8 mL of 0.125 M methanolic KOH was added to the remaining 200 μL and the mixture was heated at 37ºC for 75 min. After cooling to room temperature, 1400 μL of $CHCl_3$ (to make a 2:1 ratio to MeOH), 200 μL of 0.3 M HCl and 400 μL of a 1 M NaCl/5 % glycerol solution was added. After centrifugation for 10 minutes at 3000 rpm, the organic phase was transferred to a new tube, dried as before, and stored at -20ºC until ready for HPLC analysis.

Sphingolipids were labeled with *ortho*-phthalaldehyde (OPA) and quantified by HPLC using established protocol (Merrill et al., 2000) with L-*threo*-dihydrosphingosine as an internal standard to control for variations in extraction efficiency. Lipid pellets were dissolved in 100 μL MeOH. 50 μL of fresh OPA reagent was added to 50 μL of lipid extract solution and allowed to react for 20 min in the dark at room temperature. 300 μL column buffer (90:10 MeOH:5 mM $KHPO_4$ pH 7.0) was added and loaded onto the HPLC. Total inorganic phosphate was determined using a modification of a published method.(Merrill et al., 2000) Dried extracts of total lipids were dissolved in 0.6 mL ashing buffer (10N H_2SO_4:70% $HClO_4$:H_2O 9:1:40) and heated at 160ºC until 100 μL remained. To this was added 0.9 mL H_2O, 0.5mL 0.9% (w/v) ammonium molybdate and 0.2 mL 9% (w/v) ascorbic acid with vortexing between each addition. This mixture was incubated for 30 min at 45ºC and inorganic phosphate was determined by measuring absorption at 820 nm. For individual experiments, each data point was done in duplicate and the error bars represent ±1 SE. Representative experiments are shown. Paired student t-tests were performed using Excel only when the difference between two means was small or significance is not easily inferable by error bars.

Preliminary ceramidase assays. Ceramidase activity was determined by the release of NBD-labeled fatty acid from the fluorescent substrate NBD-C_{12}-phytoceramide. Briefly, 20 μL of total membrane preps

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were resuspended in buffer B (25 mM Tris-HCl, at three different pHs 8.56, 9.4 and 7.0, and 0.5 mM $CaCl_2$) and mixed with 20 µL of substrate dissolved in buffer B with 0.4 % Nonidet P-40). After incubation at 30^{o}C for 60 min, the reactions were stopped by boiling for 5 min and dried in a heating block at 80^{o}C for 20 min in the dark. Reaction mixtures were dissolved in 30 µL of chloroform:methanol (2:1, v/v). Twenty-five µL (25 ml) of each sample was applied to a silica gel 60A TLC plate (Whatman) and resolved by a solvent system composed of chloroform:methanol:4.2 N ammonium hydroxide (90:30:0.5, v/v/v). NDB-C_{12}-phytoceramide and NBD-dodecanoic acid were run as controls. Fluorescent lipids were detected by scanning the TLC plate in a Storm^{TM} 860 Chromatoscanner (Molecular Dynamics), operated in the fluorescent mode, with an excitation wavelength of 450 nm and an emission of wavelength of 525 nm.

Sequence analysis. Multiple sequence alignments were produced using ClustalX (Thompson et al., 1997) with default parameters. Alignments shown in **Supplemental Figures 1-3** were performed manually. Hydropathy plots were first generated with TopPredictII 1.2 (Claros and von Heijne, 1994) using the Kyte-Doolittle algorithm and default parameters. Data was downloaded into a spreadsheet and the hydropathy values were aligned based on the multiple sequence alignment produced by ClustalX. Average hydropathy across the entire set of aligned proteins was then calculated. Sequences used for these comparisons are listed in the **Supplemental Data 1**.

Results

Izh2p activation by PR-5 defensins. Before communicating the results, it is important to recap what is known about the link between Izh2p and *FET3*. *FET3* encodes a ferroxidase involved in iron-uptake whose expression is induced by iron-limitation. We previously discovered that Izh2p overexpression inhibits the ability to induce *FET3* expression during iron-limitation and that this effect is not seen in strains lacking an isoform of cAMP-dependent protein kinase (PKA, Tpk2p) or either of a pair of transcriptional repressors called Nrg1p and Nrg2p (Kupchak et al., 2007).

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These published experiments involved overexpression of the Izh2p receptor, not its activation. We interpreted the data to mean that Izh2p, like many other signaling proteins, has basal signaling activity. Herein, we confirm that Izh2p overexpression and Izh2p activation are mechanistically similar at low concentrations of agonist. Since Izh2p is activated by the plant protein osmotin, it follows that osmotin should repress *FET3* in an Izh2p-dependent manner when Izh2p is expressed at low levels. Unfortunately, osmotin is not commercially available. Thaumatin, on the other hand, is a PR-5 defensin from the plant, *Thaumatococcus daniellii*, that is sold as an industrial sweetener (Min et al., 2004). A pairwise comparison of the osmotin and thaumatin mature peptides using Blast 2 Sequences (Tatusova and Madden, 1999) reveals an expect value of $1x10^{-59}$, confirming that the two proteins are closely related. Therefore, we decided to test if thaumatin could substitute for osmotin as an activator of Izh2p.

When thaumatin is added to wild type cells carrying only the genomic copy of *IZH2*, there is a dose-dependent repression of *FET3* as shown by a reduction in cell surface ferroxidase activity (**Figure 1A**, white squares). The ED_{50} for this effect is approximately 3.6 μM. When Izh2p dosage is reduced by deleting the *IZH2* gene, *FET3* expression is desensitized to the effects of thaumatin (ED_{50} ~ 8.1 μM, **Figure 1A**, black squares) The dosage of *IZH2* can be increased by transforming wild type cells with a plasmid bearing the *IZH2* gene driven by the *GAL1* promoter. When these cells are grown in 2% galactose, Izh2p is fully induced and *FET3* is constitutively repressed (Kupchak et al., 2007). However, when these cells are grown in 0.05% galactose/1.95% raffinose, Izh2p expression is increased above normal, but not high enough to constitutively affect *FET3*. Under these latter conditions, where Izh2p levels are slightly elevated, *FET3* expression is sensitized to the effects of thaumatin (ED_{50} ~ 0.1 μM, **Figure 1A**, white triangles) Moreover, the repression of *FET3* caused by 500 nM thaumatin in cells carrying slightly elevated levels of Izh2p did not occur in strains lacking Tpk2p, Nrg1p or Nrg2p (**Figure 1B).**

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These findings indicate that Izh2p partially mediates the effects of thaumatin on *FET3*. This is consistent with the fact that the antifungal effects of osmotin, another PR-5 defensin, are only partially mediated by Izh2p (Narasimhan et al., 2005) and with a model in which Izh2p functions as a general PR-5 defensin receptor rather than as a specific osmotin receptor. More importantly, these data confirm that Izh2p overexpression and Izh2p activation repress *FET3* by similar mechanisms. However, they also show that we cannot use thaumatin treatment as a model for Izh2p activation because thaumatin clearly has Izh2p-independent effects. For this reason, unless otherwise noted, we will use full Izh2p overexpression (in 2% galactose) instead of activation as a model to isolate the effects of this receptor on downstream signaling pathways.

The PAQR family of receptors shares similarity with alkaline ceramidases. The PAQR family can be divided into three classes based on sequence alignments, however, the entire family is unified by the presence of a core of 7 transmembrane domains (TMs) (**Figure 2A**) and three highly conserved, albeit short, amino acid motifs (Lyons et al., 2004). A multiple sequence alignment of the regions containing these motifs can be found in **Supplemental Figures 1, 2** and **3**. Motif A consists of $Ex_{2\text{-}3}Nx_3H$ and can be found N-terminal to TM1. Motif B consists of an Sx_3H motif at the end of TM2 and an aspartic acid residue at the beginning of TM3. Motif C consists of an Hx_3H motif in the loop region between TM6 and TM7. **Figure 2C** shows the predicted topology of the PAQR family and the location of these motifs.

Searches of the NCBI database using the iterative PSI-BLAST program (Altschul and Koonin, 1998) revealed that PAQRs display distant similarity with a family of proteins known as alkaline ceramidases (AlkCer), although expect values for pairwise comparisons between PAQRs and AlkCers are always below the default cutoff value for significance. Like the PAQR family, the AlkCer family is unified by the presence of 7 TMs (**Figure 2B**) and three short amino acid motifs that are nearly identical in sequence and position to those that unify the PAQRs. In fact, the only difference between the consensus sequences that unify the AlkCer family from those that unify the PAQR family is the substitution of a histidine in

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motif A of the PAQRs with an asparagine in the alkaline ceramidases (**Figure 2C** and **Supplemental Figures 1, 2** and **3**).

Since Izh2p bears sequence similarity with enzymes in the alkaline ceramidase family, we decided to test if overexpression of a known alkaline ceramidase would have a similar effect on *FET3* as Izh2p overexpression. **Figure 2D** shows that, overexpression of a yeast alkaline ceramidase, Ypc1p, repressed *FET3-lacZ.* As with Izh2p overexpression or activation with thaumatin, the repression of *FET3-lacZ* by Ypc1p overexpression required Tpk2p and the Nrg1p/Nrg2p transcriptional repressors (**Figure 2D**).

Accumulation of sphingoid base in response to Izh2p overproduction. Alkaline ceramidases produce sphingoid bases from the hydrolysis of ceramides. This led us to hypothesize that sphingoid bases may be produced when Izh2p is overexpressed and play a role in the repression of *FET3*. To test this hypothesis, we measured the effect of Izh2p on steady state accumulation of the primary sphingoid base in yeast - phytosphingosine (PHS). Empty expression vector was used as a negative control and Ypc1p, whose overexpression is known to enhance PHS accumulation (Mao et al., 2000a), was used as a positive control. Overexpression of either Izh2p or Ypc1p caused an increase in the mean steady state levels of PHS in a wild type strain grown in synthetic defined medium (**Figure 3A**). We used a paired t-test to determine the significance of the difference between the control mean and the Izh2p-overexpression mean and found that the increase, although small, was significant to > 95% confidence.

It follows that, if Izh2p or Ypc1p overexpression represses *FET3* by increasing PHS levels, then the addition of exogenous PHS should bypass the need for Izh2p and directly repress *FET3*. Indeed, the addition of 100 μM PHS repressed *FET3-lacZ* (**Figure 3B**). The addition of a similar lipid (stearylamine) at the same concentration had no effect on *FET3-lacZ* suggesting that the repression of *FET3* by PHS was not due to a non-specific effect of the lipid on membranes. As with other treatments, the repression of *FET3-lacZ* by exogenous PHS required Tpk2p and the Nrg1p/Nrg2p transcriptional repressors (**Figure 3C**).

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Thus, it is clear that Izh2p increases steady state levels of PHS and that PHS is capable of mediating the repression of *FET3* in a manner that requires the same signaling proteins as Izh2p overexpression. These findings suggest that any treatment that causes PHS to accumulate would have a similar effect on *FET3* expression. One simple way to elicit PHS accumulation is by preventing its catabolism. This can be achieved by adding N,N-dimethylsphingosine (N,N-DMS), a specific inhibitor of the first step (phosphorylation) of sphingoid base degradation (Welsch et al., 2004). Alternatively, the prevention of ceramide synthesis also results in PHS accumulation. This can be achieved by adding fumonisin B_1 (FB_1), a specific inhibitor of the acyl-CoA-dependent ceramide synthases (Wu et al., 1995). Both N,N-DMS and FB_1 cause a dose dependent repression of *FET3-lacZ* in wild type cells carrying empty expression vector (**Figure 3D**).

The effect of Izh2p requires ceramide biosynthesis but does not require alkaline ceramidases. The data in the previous sections suggest that the effect of Izh2p on *FET3* is mediated by PHS, which raises the question of how Izh2p affects PHS levels. One possibility is that Izh2p stimulates an endogenous ceramidase enzyme and thus enhances ceramide catabolism. Yeast only possess two known ceramidases (Ypc1p and Ydc1p), both of which belong to the alkaline ceramidase family (Mao et al., 2000b). The logical experiment is to see if Izh2p overexpression still elicits PHS accumulation in a strain lacking Ypc1p and Ydc1p (*ypc1*Δ*ydc1*Δ). Indeed, Izh2p overexpression still elicits significant PHS accumulation in this double mutant strain (**Figure 4A**). Overexpression of Ypc1p in *ypc1*Δ*ydc1*Δ is used as a positive control. A paired t-test comparing the control and Izh2p-overexpressor means indicates that the increase in PHS elicited by Izh2p is small, but significant to > 95% confidence. These results indicate that Izh2p does not increase PHS by stimulating the activity of known ceramidases.

Another possibility is that Izh2p stimulates PHS biosynthesis or prevents its incorporation into ceramides. To distinguish between these possibilities, we performed two important experiments. First, we measured the effect of short term FB_1 treatment on Izh2p-dependent PHS accumulation in the *ypc1*Δ*ydc1*Δ strain.

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Treatment with FB_1 prevents the incorporation of newly made PHS into ceramides by inhibiting the CoA-dependent ceramide synthases. While FB_1 treatment (at a concentration that maximally represses *FET3-lacZ*) causes a large accumulation of PHS in all strains (data not shown), there is no further increase in PHS accumulation in cells overexpressing Izh2p relative to those carrying empty expression vector. This is evident in **Figure 4B**, which plots the ratio of PHS accumulation in Izh2p-overexpressing cells over PHS accumulation in cells carrying empty expression vector. The second experiment measured the effect of the antifungal drug, myriocin, under the same conditions. Myriocin is a specific inhibitor of serine palmitoyltransferase and, as such, stops *de novo* sphingoid base biosynthesis (Yamaji-Hasegawa et al., 2005). **Figure 4B** shows that short-term treatment with myriocin also prevented the accumulation of PHS caused by Izh2p overexpression. Moreover, myriocin caused a dose-dependent alleviation of the repression of *FET3-lacZ* caused by Izh2p overexpression (**Figure 4C**). Note that the concentration of myriocin that maximally represses Izh2p-dependent *FET3-lacZ* repression fully suppresses PHS accumulation caused by Izh2p overexpression. (At very high concentrations of myriocin, we saw a decrease in *FET3-lacZ* even in cells carrying empty expression vector. This effect is likely due to the fungicidal properties of myriocin at these concentrations.) Other means of repressing *FET3-lacZ*, such as *YPC1* overexpression, treatment with 100 μM PHS or the addition of 1 mM Fe^{3+} to the grown medium were unaffected by short term treatment with myriocin (**Figure 4C**).

Testing the ceramidase activity of Izh2p. One explanation for these data is that Izh2p functions as an alkaline ceramidase that hydrolyzes newly made ceramides. The simplest experiment to test this model would be to purify Izh2p and measure its ability to hydrolyze ceramide substrates. However, attempts to purify and reconstitute Izh2p have not yet been successful. To get around this problem, we measured ceramidase activity in total membrane preparations from cells carrying either empty vector or the Ypc1p and Izh2p ovexpression plasmids. The substrate for this assay was phytosphingosine that had been N-acylated with fluorescently labeled dodecanoic acid. After exposing the substrate to membrane preps for 1 hour at various pH values, we did not see any bands on the TLC plate that corresponded to the free fatty acid in the Izh2p overexpressing lane (data not shown). While this result is difficult to reconcile with the

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proposed ceramidase function for Izh2p, it is important to note that we have serious reservations about the validity of this experiment that will be addressed in the discussion.

Despite the negative result from the *in vitro* ceramidase assays, we decided to see if D-*erythro*-MAPP, an inhibitor of alkaline ceramidases (Szulc et al., 2007), could inhibit Izh2p-dependent repression of a *FET3-lacZ* reporter construct. Consistent with our model, D-*erythro*-MAPP alleviated the effects of both Izh2p overproduction (in 2% galactose) and activation (in 0.05% galactose + 500 nM thaumatin) on *FET3-lacZ* expression with ED_{50} values of ~ 1 nM and 5 nM, respectively (**Figure 5A**). Moreover, D-*erythro*-MAPP also potently inhibited Ypc1p-dependent *FET3* repression (ED_{50} ~ 150 pM). D-*erythro*-MAPP had no effect on *FET3-lacZ* in strains carrying empty expression vector or in cells where *FET3-lacZ* is repressed by exogenous PHS, indicating that PHS functions downstream of D-*erythro*-MAPP in the pathway. In addition, the repression of *FET3-lacZ* by high levels of iron was not alleviated by D-*erythro*-MAPP, indicating that ceramidase inhibition does not result in general induction of *FET3* (**Figure 5A**).

Izh2p confers fumonisin B_1 resistance. The acyl-CoA-dependent ceramide synthases are sensitive to fumonisin B_1 (FB_1) and treatment with this drug results in decreased accumulation of complex sphingolipids (Cowart and Obeid, 2007). Since complex sphingolipids are essential for yeast, the sensitivity of yeast to FB_1 has been used to identify genes that can bypass the need for acyl-CoA-dependent ceramide synthesis in the production of ceramides. In fact, this screen led to the discovery of the alkaline ceramidases, which have FB_1-independent ceramide synthase or "reverse ceramidase" activity (Mao et al., 2000a). In **Figure 5B**, we show that Izh2p overexpression resulted in the same level of resistance to FB_1 as does Ypc1p overexpression, suggesting that it, too, can overcome the ceramide synthase defect.

Izh2p- and sphingoid base dependent *FET3* repression requires Pkh1p and Pkh2p. If sphingoid base is the second messenger generated by Izh2p, what transduces the message further downstream? Intriguingly, the *IZH1* and *IZH4* genes are divergently transcribed from the *PKH1* and *PKH2* genes,

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respectively (Lyons et al., 2004). The organization of these genes has long led us to suspect the involvement of the *PKH* gene products in *IZH*-dependent signaling. *PKH1* and *PKH2* genes encode a pair of partially redundant kinases and deletion of either *PKH1* or *PKH2* resulted in loss of repression of *FET3-lacZ* in response to Izh2p overexpression, Ypc1p overexpression or treatment with exogenous PHS (**Figure 6A**). Ypc1p overexpression and PHS treatment still exerted small effects in the *pkh1*Δ and *pkh2*Δ mutants, presumably due to the redundant functions of Pkh1p and Pkh2p. Since the *pkh1*Δ*pkh2*Δ double mutation is lethal (Casamayor et al., 1999), we could not measure the effect of losing both kinases on Izh2p-dependent *FET3* repression. Pkh1p and Pkh2p were also essential for *FET3-lacZ* repression caused by thaumatin activation of slightly elevated levels of Izh2p (in 0.05% galactose) (**Figure 6B**). It must be noted that *FET3-lacZ* and cell surface ferroxidase activities responded normally to iron in the *pkh1*Δ and *pkh2*Δ strains indicating that these mutations did not result in a generalized inability to repress *FET3* (data not shown).

Discussion

Data from this other published work (Kupchak et al., 2007; Smith et al., 2008) allow us to draw some important conclusions about how Izh2p might work as a receptor. For example, the ED_{50} for thaumatin-dependent *FET3* repression is decreased by increasing the expression of Izh2p. This can be explained by a synergism between the Izh2p-dependent and Izh2p-independent pathways of signal transduction. However, these data also fit the spare receptors model (for review, see Zhu, 1993), which would suggest that the amount of signal produced by Izh2p exceeds the capacity of the downstream signaling pathway. In other words, there are either more Izh2p or more second messengers produced by Izh2p than there are effectors to carry the signal further downstream.

The fact that Izh2p can overwhelm downstream effectors helps explain our finding that Izh2p (as well as several human PAQRs; see Kupchak et al., 2007; Smith et al., 2008) constitutively transduce signals when expressed at high levels but require agonist when expressed at low levels. One interpretation of this

finding is that there is a "signal transduction repressor" found in excess of Izh2p and that this repressor can be dissociated by agonist binding at low receptor concentrations or titrated away by receptor overproduction. However, the spare receptors model argues against there being such a "signal repressor" since its existence would imply a signaling bottleneck at the receptor and not downstream of it. An alternative interpretation is that, under normal conditions, Izh2p (and perhaps other PAQRs) constitutively produces very low quantities of a second messenger that are too low to activate downstream effectors. When Izh2p is activated or overexpressed, the net production of messenger rises above the threshold necessary to saturate downstream effectors.

What remains to be determined is the chemical identity of this second messenger and, to achieve this, we decided to map the signal transduction pathway using the repression of *FET3* as a reporter of receptor activity. This is where the use of yeast as a model system has been particularly fruitful. Our previous studies showed that Izh2p regulated *FET3* by affecting a competition between the Msn2p/Msn4p transcriptional co-activators and the Nrg1p/Nrg2p transcriptional repressors, which have opposing effects on an element in the *FET3* promoter (Kupchak et al., 2007).

The identification of these transcription factors allowed us to begin mapping the signal transduction pathway from the bottom-up. Msn2p/Msn4p are negatively regulated by PKA, which causes their translocation into the cytoplasm (Garmendia-Torres et al., 2007). Furthermore, Nrg1p/Nrg2p are negatively regulated by nuclear AMPK (Vyas et al., 2001) and active PKA causes AMPK to relocalize to the cytoplasm (Hedbacker et al., 2004). Thus, the balance of the competition between Nrg1p/Nrg2p and Msn2p/Msn4p can be tipped towards Nrg1p/Nrg2p, and therefore *FET3* repression, by simply activating PKA. Not surprisingly, PKA, Nrg1p and Nrg2p are all essential for *FET3* repression caused by either Izh2p overexpression, Izh2p activation, Ypc1p overexpression or PHS treatment. A model for this signal transduction pathway is shown in **Figure 7**, although it must be noted that much more work is required to confirm this model.

The problem with this bottom-up approach to mapping the Izh2p-dependent signaling pathway is that it does not connect PKA to Izh2p. To make this connection, it is necessary to map the pathway from the top-down. Unfortunately, Izh2p is not known to interact physically or genetically with other proteins, making it difficult to develop models for how it might activate PKA. However, the fact that Izh2p resembles a group of proteins with a known enzymatic activity gave us a starting point. While it is uncertain if the similarities between the PAQR and alkaline ceramidase families are the result of random chance, common ancestry or convergent evolution, it is intriguing that overexpression of Ypc1p has the same effect on *FET3* as Izh2p overexpression or activation. It is also intriguing that both Izh2p activation and Ypc1p overexpression have both been shown to induce apoptosis in yeast (Aerts et al., 2008; Narasimhan et al., 2005), indicating that the functional link between Izh2p and Ypc1p is not merely an artifact of our reporter system.

Since alkaline ceramidases produce PHS, we hypothesized that Izh2p also produces PHS. Indeed, Izh2p overexpression does cause a small but significant increase in steady state levels of PHS. It is important to note that the relatively small increase total PHS caused by Izh2p is likely to be a reflection of Izh2p affecting a distinct pool of PHS that may be small when compared to bulk PHS. Indeed, the fact that myriocin inhibits Izh2p-dependent but not Ypc1p-dependent *FET3* repression suggests that Izh2p and Ypc1p affect different pools of PHS.

The inhibition of Izh2p-dependent signaling by myriocin suggests that Izh2p stimulates *de novo* PHS biosynthesis. However, the fact that FB_1 can prevent Izh2p-dependent PHS accumulation suggests that the pool of PHS that is influenced by Izh2p must first be incorporated into ceramides. By corollary, this finding also suggests that these newly generated ceramides must be hydrolyzed to produce the PHS we measured. We know, however, that Izh2p-dependent accumulation of PHS does not require the known endogenous ceramidases, Ypc1p and Ydc1p. This leaves us with the proposition that Izh2p is stimulating some ceramidase activity that is independent of the known enzymes. Of course, the similarities between

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the PAQRs and alkaline ceramidases make it enticing to speculate that Izh2p, itself, possesses an intrinsic ceramidase activity.

We have attempted to test this idea by assaying ceramidase activity in membrane from cells overexpressing Izh2p and found no increase in the ability to hydrolyze a fluorescently labeled synthetic ceramide substrate. However, it must be stated that it is extremely difficult to interpret this result. For example, it is possible that the synthetic substrate we chose is simply not a good substrate for Izh2p. After all, there are dozens of ceramide permutations that differ in head group, fatty acid and sphingoid base. The lack of reactivity in our experiment could merely be due to the bulky fluorescent label or to the absence of the correct head group or fatty acid. Moreover, it is possible that Izh2p requires some soluble cofactor or subunit that did not purify with our membranes. Consequently, this line of experimentation is premature and what is needed is a sphingolipidomic study to identify particular ceramide species that may be altered by Izh2p overexpression in order to narrow down the search for potential substrates.

For now, we have presented some circumstantial evidence that Izh2p may, indeed, function as an alkaline ceramidase. First, Izh2p-dependent repression of *FET3* was potently inhibited by D-*erythro*-MAPP, a known inhibitor of alkaline ceramidases. It should be noted, however, that there are concerns about the specificity of D-*erythro*-MAPP and it is possible that this drug exerts its effects on Izh2p-dependent signaling by an off-target mechanism. Second, Izh2p rescued the FB_1-sensitivity of a *yor1*Δ strain suggesting that this protein has "reverse ceramidase" or ceramide synthase activity, a hallmark of alkaline ceramidases. Taken together, these results suggest that Izh2p may possesses ceramidase activity, although, much more work needs to be done before this assertion can be proven. This line of questioning will be the focus on future research.

In the meantime, our data also provides an explanation for how sphingoid bases might affect PKA activity, thus connecting our top-down and bottom-up approaches to mapping the signal transduction pathway. We demonstrated that the kinases (Pkh1p and Pkh2p) are essential for Izh2p- and sphingoid

base-dependent repression of *FET3*. These kinases are homologs of human PDK1 (Inagaki et al., 1999), which functions as a master regulator of downstream kinases in the AGC kinase family (Komander et al., 2005). The potential involvement of the yeast PDK1 homologs is intriguing for several reasons. First, PDK1, Pkh1p and Pkh2p are directly activated by sphingoid bases (Friant et al., 2001; Liu et al., 2005). Second, PDK1 homologs in both humans and fungi have been shown to activate PKA (Cheng et al., 1998; Tang and McLeod, 2004). Third, yeast Pkh1p was found to physically interact with an isoform of PKA in a 2-hybrid screen (Ho et al., 2002). Fourth, there is evidence from mammalian cells suggesting that sphingoid base activates PKA by mimicking the positive effects of cAMP on its regulatory subunit (Ma et al., 2005).

Hence, we propose a model in which Izh2p or a yet-to-be identified protein regulated by Izh2p, catabolize newly made ceramides to generate sphingoid base. The accumulation of sphingoid base activates the yeast homologs of PDK1, which, in turn, activate PKA. The activation of PKA then regulates *FET3* expression through its ability to sequester AMPK and Msn2p/Msn4p in the cytoplasm. Of course, more work is necessary to unequivocally confirm this model, however, it is consistent with our data and what is known the signaling proteins involved. It is also intriguing to note that sphingolipids have been shown to function downstream of both adiponectin and progesterone receptors (Kase et al., 2007; Strum et al., 1995), suggesting that the human PAQRs also transduce signals by the same mechanism. Future studies will focus on determining if human PAQRs also produce sphingoid base second messengers.

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Footnotes

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Figure Legends

Figure 1. Thaumatin represses *FET3* expression via Izh2p. In panels **(A)** and **(B)**, cells were grown in iron-limited LIM containing 0.05% galactose to modestly overexpress *IZH2* driven by the *GAL1* promoter. Cell surface ferroxidase activities are plotted as a percentage of activity seen in wild type cells carrying empty expression vector. **(A)** WT (BY4742) or isogenic *izh2*Δ mutant cells carry either empty expression vector (white, black squares) or a plasmid that overexpresses *IZH2* (white triangles). **(B)** Ferroxidase activity generated by the *FET3* gene shown as a percentage of activity seen in untreated WT (BY4742) cells carrying the *IZH2* overexpression vector. All strains carry the *IZH2* overexpression plasmid and were either left untreated (white bars) or exposed to 500 nM thaumatin (grey bars). For all panels, error bars indicate ±1 SE (n=3). Δ symbols indicate isogenic strains lacking particular genes.

Figure 2: Hydropathy plots of PAQRs receptors and alkaline ceramidases. In panels **(A)** and **(B)**, hydropathy plots for individual proteins were generated and aligned (dotted lines). An average hydropathy plot for all proteins plotted in each panel was generated (solid line). The locations of conserved motifs (designated by a circled letter) are shown with letters and the predicted TMs are numbered. The sequences used to generate these figures are listed in the Supplemental Data. **(A)** Various fungal members of the PAQR family. **(B)** Various members of the alkaline ceramidase family. **(C)** Predicted topology of the PAQR and alkaline ceramidase families with the locations and consensus sequences of the three highly conserved motifs. **(D)** WT (BY4742) and isogenic mutant cells were grown in iron-limited LIM containing 2% galactose to overexpress *YPC1* driven by the *GAL1* promoter. β-galactosidase activity generated by the *FET3-lacZ* reporter is shown as a percentage of activity seen in wild type cells carrying empty expression vector. Δ symbols indicate strains lacking particular genes.

Figure 3. Involvement of sphingoid bases in Izh2p-dependent signaling. In all panels, cells were grown in iron-deficient LIM containing 2% galactose to induce *GAL1* driven genes. **(A)** Steady state

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levels of phytosphingosine in WT (BY4742) cells carrying either empty expression vector, the *YPC1* overexpression vector or the *IZH2* overexpression vector. Asterisk indicates p value that is statistically significant at the 95% confidence interval. Dotted lines indicate the populations compared using the t-tests. Error bars indicate ±1 SE (n=2). **(B)** The addition of 100 μM exogenous phytosphingosine (PHS) or stearylamine to WT (BY4742) cells carrying empty expression vector represses *FET3-lacZ*. Untreated cells were actually treated with an equivalent volume of ethanol to control for vehicle effects. Error bars indicate ±1 SE (n=3). **(C)** The repression of *FET3-lacZ* caused by treatment of cells with 100 μM PHS was alleviated in *tpk2*Δ, *nrg1*Δ and *nrg2*Δ mutant strains isogenic to BY4742 WT. Error bars indicate ±1 SE (n=3). Δ symbols indicate strains lacking particular genes. **(D)** FB_1 and N,N-DMS repress *FET3-lacZ* in a dose dependent manner in WT (BY4742) cells carrying empty expression vector. Error bars indicate ±1 SE (n=3).

Figure 4. Izh2p-dependent signaling does not require endogenous alkaline ceramidases. (A) Steady state accumulation of phytosphingosine in *ypc1*Δ*ydc1*Δ double mutant cells isogenic to WT (W303-1A) carrying either empty expression vector or the *YPC1* and *IZH2* overexpression plasmids grown in synthetic defined medium with 2% galactose. Asterisk indicates p value that is statistically significant at the 95% confidence interval. Dotted lines indicate the populations compared using the t-tests. Error bars indicate ±1 SE (n=2). **(B)** Steady state accumulation of phytosphingosine in *ypc1*Δ*ydc1*Δ mutant cells isogenic to WT (W303-1A) carrying either empty expression vector or the *IZH2* overexpression plasmid grown in synthetic defined medium with 2% galactose. Cells were either left untreated or treated for 30 min with 100 μM FB_1 or 1 μM myriocin. Error bars indicate ±1 SE (n=2). **(C)** β-galactosidase activity from *FET3-lacZ* is plotted as a percentage of activity seen in untreated wild type cells carrying empty expression vector as a control. WT (BY4742) cells were grown in iron-deficient LIM containing 2% galactose. The effect of 1 hr treatment with myriocin on *lacZ* activity in wild type cells carrying either empty expression vector (squares), the *IZH2* overexpression plasmid (circles) or the *YPC1* overexpression

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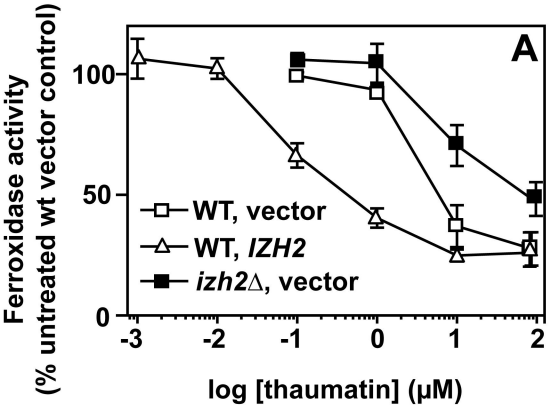
plasmid (triangles). Control cells carrying empty expression vector were also treated with 100 μM phytosphingosine (PHS, diamonds) or 1 mM Fe^{3+} (black squares). Error bars indicate ±1 SE (n=3).

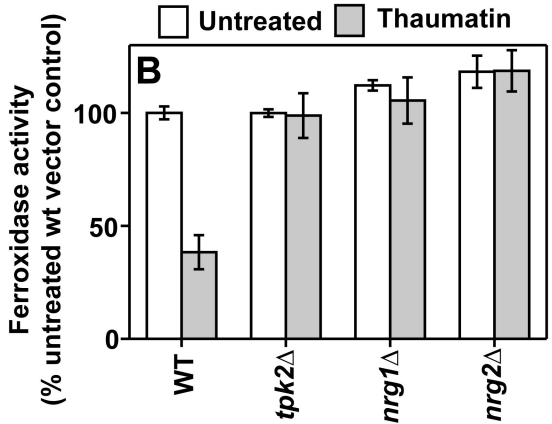
Figure 5. Possible ceramidase function for Izh2p. (A) β-galactosidase activity generated by the *FET3-lacZ* reporter is shown as a percentage of activity seen in untreated WT (BY4742) cells carrying the empty expression vector. Cells were grown in iron-deficient LIM containing either 2% galactose (white, black symbols) or 0.05% galactose + 500 nM thaumatin (grey symbols). The effect of treatment with D-*erythro*-MAPP on *lacZ* activity in wild type cells carrying either empty expression vector (squares), the *IZH2* overexpression plasmid (diamonds) or the *YPC1* overexpression plasmid (triangles). Controls show that D-erythro-MAPP has no effect on *FET3-lacZ* repression causes by either treatment with 100 μM PHS (black crosses) or 1 mM Fe^{3+} (black squares). Error bars indicate ±1 SE (n=3). **(B)** Growth curves of *yor1*Δ mutant cells isogenic to WT (BY4742) carrying either empty expression vector (squares) or the *IZH2* (diamonds) and *YPC1* (triangles) overexpression plasmids. Cells were either left untreated (grey symbols) or exposed to 100 μM FB_1 (white symbols). Error bars indicate ±1 SE (n=3).

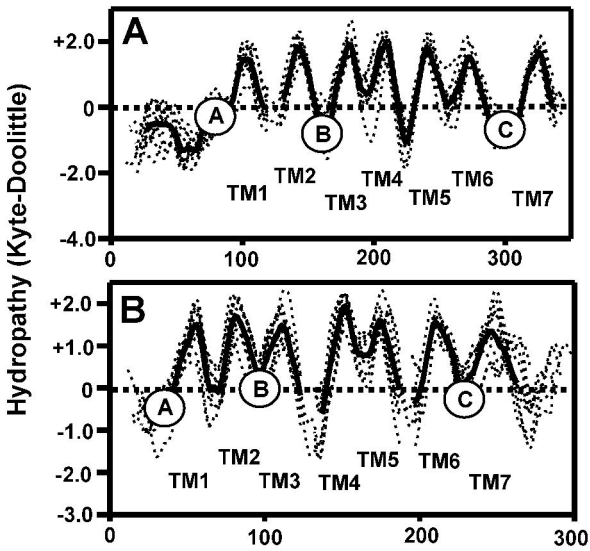
Figure 6. Yeast phosphoinositide-dependent kinases are involved in Izh2p-dependent signaling. (A) β-galactosidase activity from *FET3-lacZ* is plotted as a percentage of activity seen in untreated WT cells carrying empty expression vector as a control. Cells were grown in iron-deficient LIM containing 2% galactose to induce *GAL1*-driven genes. *lacZ* activity in WT (BY4742) and *pkh1*Δ and *pkh2*Δ isogenic mutant strains carrying either empty expression vector (dotted white bars), the *IZH2* overexpression plasmid (white bars) or the *YPC1* overexpression plasmid (grey bars). Vector control was also treated with 100 μM PHS (black bars). Error bars indicate ±1 SE (n=3). **(B)** β-galactosidase activity from *FET3-lacZ* is plotted as a percentage of activity seen in untreated WT cells carrying empty expression vector as a control. WT (BY4742) and isogenic mutant cells were grown in iron-deficient LIM containing 0.05%

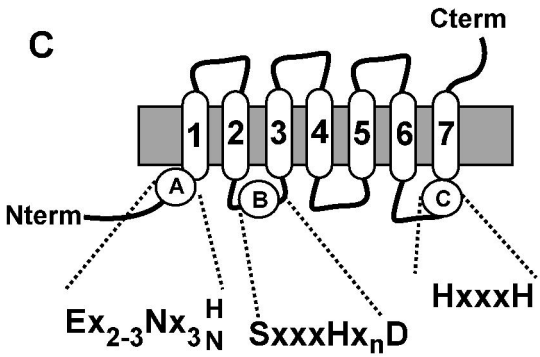
galactose to modestly induce *GAL1*-driven genes. Cells were treated with 500 nM thaumatin. Error bars indicate ±1 SE (n=3). Δ symbols in both panels indicate strains lacking particular genes.

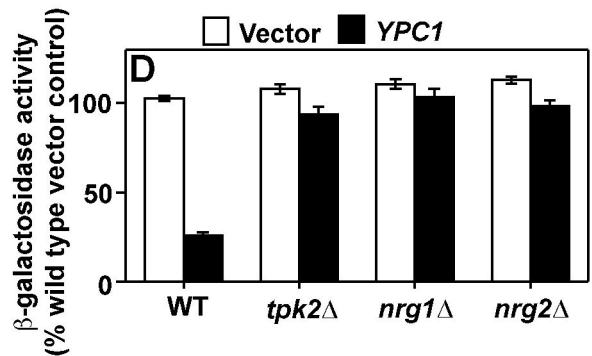
Figure 7. A model Izh2p-dependent signaling in yeast cells. Solid lines indicate that a particular molecule or protein acting upon another. Dashed lines indicate the effects. Arrows indicate positive effects while bars indicate negative effects.

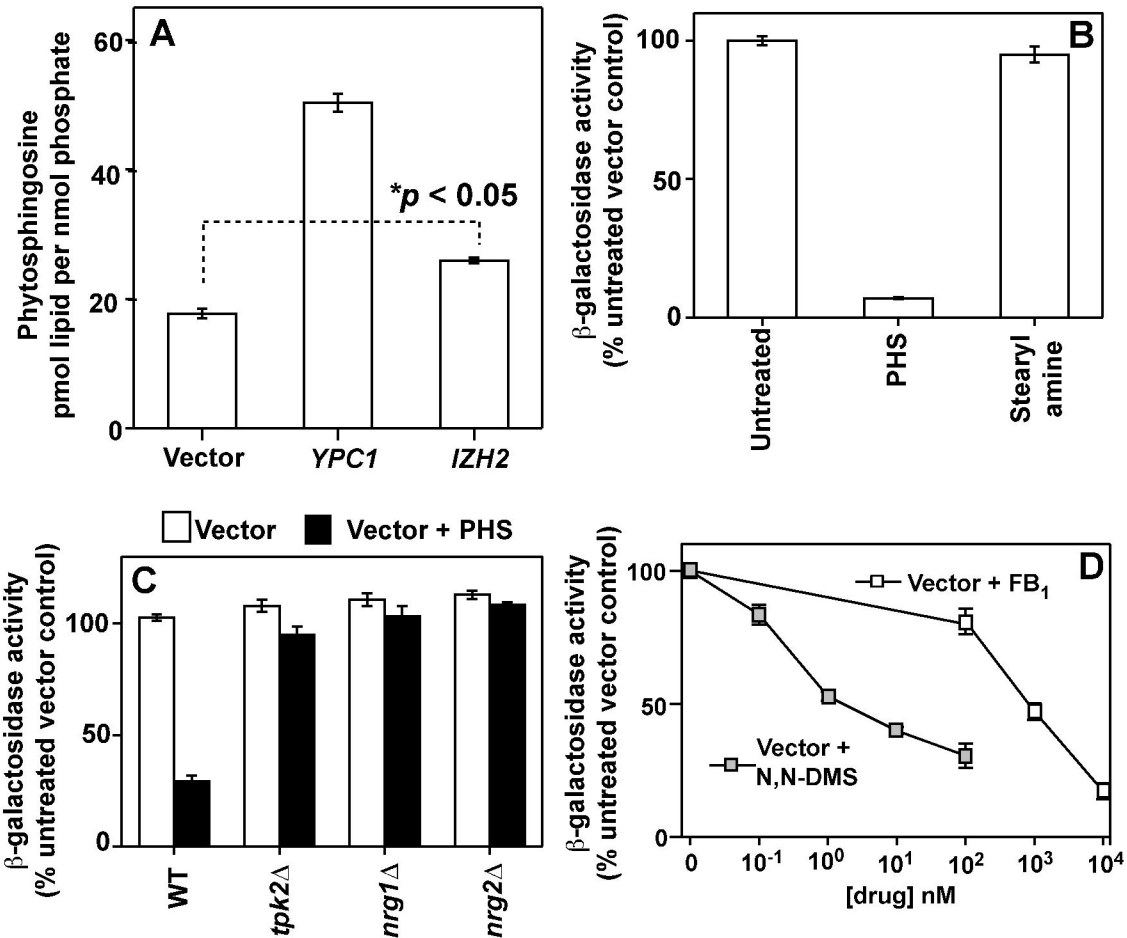

A
Ferroxidase activity
(% untreated wt vector control)
100
50
0
-3
-2
-1
0
1
2
log [thaumatin] (μM)
WT, vector
WT, IZH2
izh2Δ, vector


Untreated
Thaumatin
B
Ferroxidase activity
(% untreated wt vector control)
100
50
0
WT
tpk2Δ
nrg1Δ
nrg2Δ


A
+2.0
0
-2.0
-4.0
A
B
C
TM1
TM2
TM3
TM4
TM5
TM6
TM7
0
100
200
300
Hydropathy (Kyte-Doolittle)
B
+2.0
+1.0
0
-1.0
-2.0
-3.0
A
B
C
TM1
TM2
TM3
TM4
TM5
TM6
TM7
0
100
200
300


C
Cterm
1
2
3
4
5
6
7
A
B
C
Nterm
HxxxH
Ex2-3Nx3 H N
SxxxHxnD


Vector
YPC1
D
β-galactosidase activity
(% wild type vector control)
100
50
0
WT
tpk2Δ
nrg1Δ
nrg2Δ


A
Phytosphingosine
pmol lipid per nmol phosphate
60
40
20
0
*p < 0.05
Vector
YPC1
IZH2
B
β-galactosidase activity
(% untreated vector control)
100
50
0
Untreated
PHS
Stearyl amine
Vector
Vector + PHS
C
β-galactosidase activity
(% untreated vector control)
100
50
0
WT
tpk2Δ
nrg1Δ
nrg2Δ
D
β-galactosidase activity
(% untreated vector control)
100
50
0
Vector + FB1
Vector +
N,N-DMS
0
10-1
100
101
102
103
104
[drug] nM

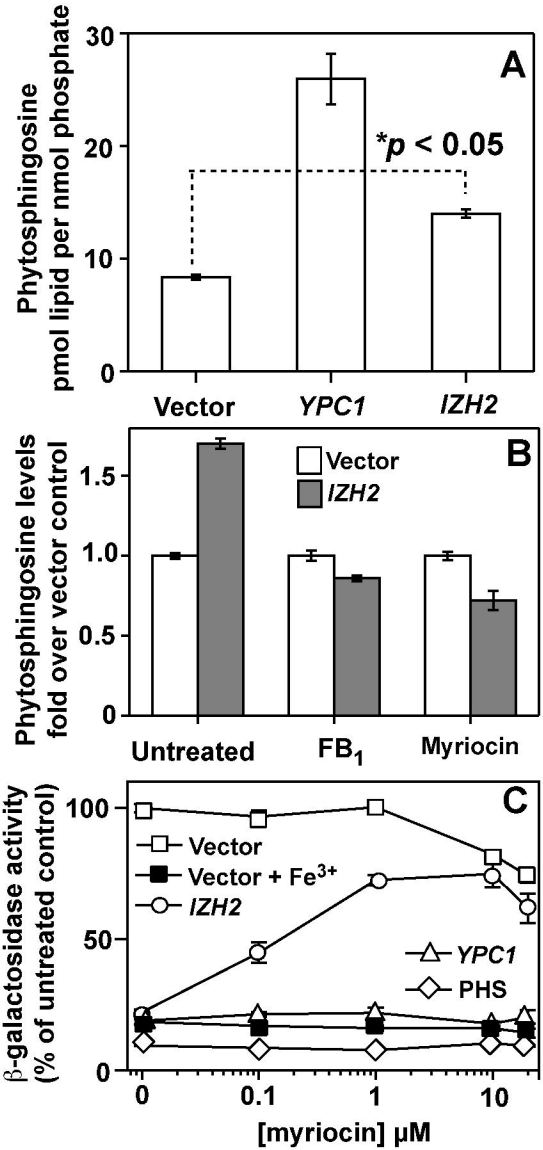
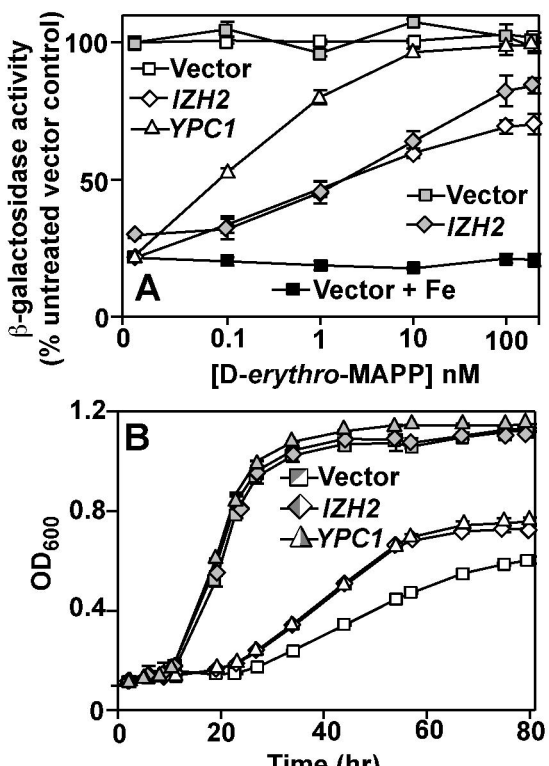

A
Phytosphingosine
pmol lipid per nmol phosphate
30
20
10
0
*p < 0.05
Vector
YPC1
IZH2
B
Phytosphingosine levels
fold over vector control
1.5
1.0
0.5
0
Vector
IZH2
Untreated
FB1
Myriocin
C
β-galactosidase activity
(% of untreated control)
100
50
0
Vector
Vector + Fe3+
IZH2
YPC1
PHS
0
0.1
1
10
[myriocin] μM

Figure 5


β-galactosidase activity
(% untreated vector control)
100
50
0
Vector
IZH2
YPC1
Vector
IZH2
A
Vector + Fe
0
0.1
1
10
100
[D-erythro-MAPP] nM
1.2
0.8
0.4
0
B
Vector
IZH2
YPC1
OD600
0
20
40
60
80
Time (hr)

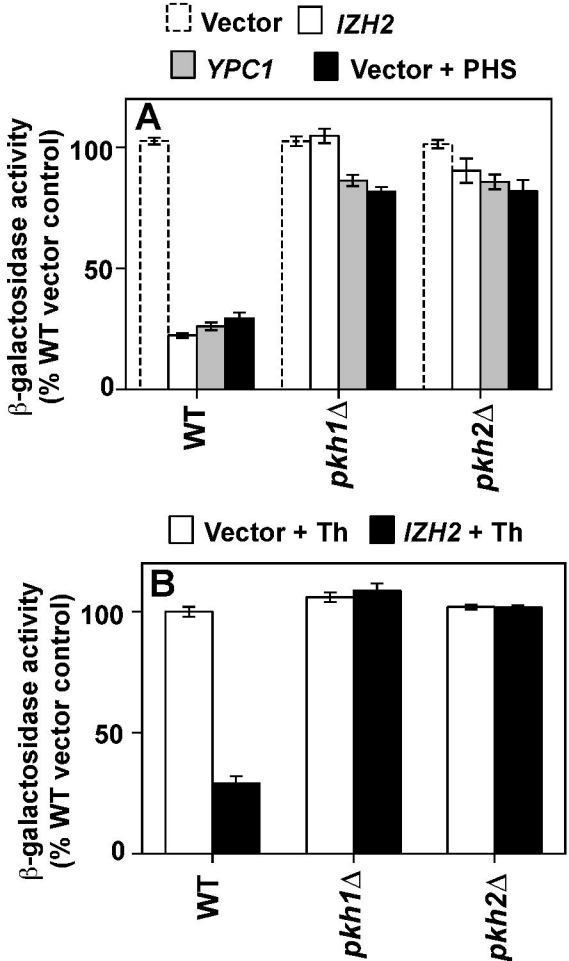
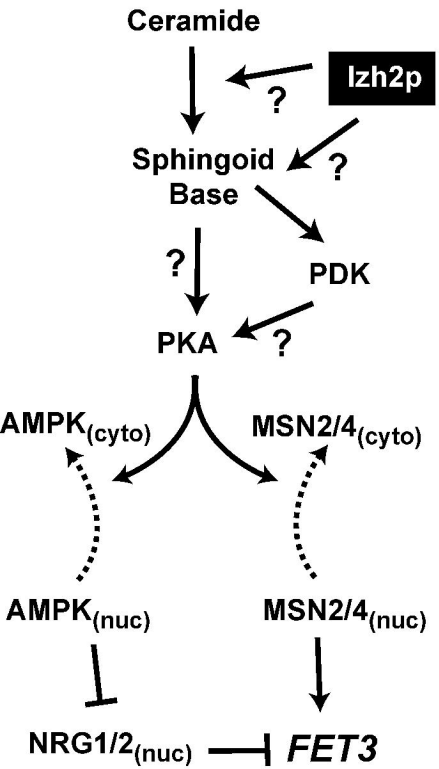

Vector
IZH2
YPC1
Vector + PHS
A
β-galactosidase activity
(% WT vector control)
100
50
0
WT
pkh1Δ
pkh2Δ
Vector + Th
IZH2 + Th
B
β-galactosidase activity
(% WT vector control)
100
50
0
WT
pkh1Δ
pkh2Δ

Figure 7


Ceramide
Izh2p
?
Sphingoid Base
?
?
PDK
PKA
?
AMPK(cyto)
MSN2/4(cyto)
AMPK(nuc)
MSN2/4(nuc)
NRG1/2(nuc)
FET3