Orm2 protein level regulates sphingolipid synthesis in response to environmental stress in yeast

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Abstract

Sphingolipids are a class of membrane lipids essential to all eukaryotic life. Found on the outer leaflet of the membrane, complex sphingolipids are a major component of microdomains, while sphingolipid metabolites and intermediates have numerous signaling roles within the cell. Despite their fundamental importance, relatively little is known regarding the specifics of sphingolipid function, or the mechanisms regulating their metabolism. In yeast, *ORM1* and *ORM2* encode proteins recently found to negatively regulate the first, and rate-limiting step in sphingolipid biosynthesis. Sphingolipid synthesis is down regulated in response to endoplasmic reticulum stress through increases in *ORM2* transcription. The Orms also respond to sphingolipid metabolites and intermediates via phosphorylation through TOR signaling, a major growth and cell cycle regulatory pathway. In this thesis, I describe two stress conditions, loss of extracellular inositol availability, and cell wall stress, both of which lead to a down regulation in sphingolipid biosynthesis via an increase in *ORM2* transcription. My findings help further elucidate the cellular and environmental cues to which cells respond by changing sphingolipid content to alter membrane fluidity and dynamics.

Introduction

The Roles of Sphingolipids

Sphingolipids are essential structural components of eukaryotic cell membranes that regulate membrane fluidity. The plasma membrane, once thought to be a relatively free-flowing bed of lipids and proteins or a “fluid mosaic”, now appears to be a far more complex and regulated organelle (Pike 2003). Much of this regulation both spatially and temporally is dependent on detergent resistant microdomains or lipid-rafts, which act as signaling platforms and allow for the preferential grouping of a variety of integral and membrane bound proteins (Pike 2003). Sphingolipids associate laterally with sterols to form microdomains on the outer leaflet of the plasma membrane (Lingwood and Simons 2010).

In addition to their structural role, sphingolipid precursors and metabolites have profound regulatory capabilities (Cowart 2006 and Nickels and Broach 1996). Phytosphingosine (PHS), one of the earliest precursors in the sphingolipid synthesis pathway activates a number of signaling cascades, many of which are still being elucidated (Cowart 2006). A later intermediate, ceramide, has a well-characterized role in activating the ceramide activated phosphatase protein (CAPP) implicated in cell cycle and growth regulation (Nickels and Broach 1996). Sphingolipid metabolites have also been shown to participate in regulation of actin polarization and endocytosis (Souza and Pichler 2012).

Sphingolipid regulation and accumulation of sphingolipid metabolites have emerging relevance in the medical fields. Recent evidence has shown links between accumulation of sphingolipid precursors and a number of diseases associated with obesity, including diabetes, cardiomyopathy, and atherosclerosis (Summers 2010). Inhibition of the first and rate-limiting step of sphingolipid synthesis has been shown to alleviate these disorders, as well as improve cardiac tissue and adipocyte morphology in rodents (Summers 2010).

Furthermore, a genetic link between development of childhood asthma and over-expression of ORMDL3 (see below) implicates sphingolipid regulation in human health and disease pathology (Wjst 2008).

Sphingolipid Synthesis

Mammals and lower eukaryotes share common steps in early sphingolipid synthesis and creation of metabolites, however yeast are more limited in their complex sphingolipid species. While mammal cells contain hundreds of unique sphingolipids and glycosphingolipids, yeast are limited to three (Futerman and Riezman 2005). The yeast sphingolipid synthesis pathway is diagrammed in Fig. 1. Enzymes catalyzing steps in sphingolipid synthesis have been identified. De novo synthesis of sphingolipids in yeast begins with the rate determining reaction in which serine and palmitoyl-CoA are condensed (Fig. 1 and Funato 2002). This reaction is catalyzed by the enzyme complex serine-palmitoyl transferase (SPT), composed of subunits *LCB1, LCB2,* and *TSC3*. This critical step is regulated by Orm1 and Orm2 (see below) and artificially inhibited by the drug myriocin (Fig. 1 and Han 2010, Breslow 2010). Downstream of SPT, the products phytosphingosine (PHS) and dihydrophingosine (DHS), often referred to as long chain base, are formed. Long chain base is then added to a very long chain base (C26 fattyacyl-CoA) by the enzyme ceramide synthase (composed of subunits *LAC1, LAG1,* and *LIP1*) to produce ceramide (Fig. 1 and Funato 2002). Further downstream, *AUR1* catalyzes the conversion from ceramide to the first of three complex sphingolipids in yeast, inositolphosphorylceramide (IPC), which has a phosphatidylinositol (PI) head group (Fig. 1 and Funato 2002). IPC is further conjugated via mannosylation to mannosylinositolphosphorylceramide (MIPC) by *CSG2/SUR1* (Fig. 1 and Funato 2002). Finally, addition of a second PI group to MIPC (catalyzed by *IPT1*) forms mannosyl(inositol)$_2$phosphorylceramide, the third of the yeast complex sphingolipids (Fig. 1 and Cowart and Obeid 2006). In yeast, inositol is the head group common to all three complex sphingolipids (Smith 1974).

Inositol Import and Synthesis

In order to synthesize the complex sphingolipids, yeast require inositol in the form of PI (Villa-Garcia 2010). Yeast cells prefer exogenous inositol to create PI and the phosphoinositides, but can also perform de novo inositol synthesis catalyzed enzymatically by *INO1* (White 1991). *INO1* encodes inositol-1-phosphate synthase, responsible for catalyzing the first committed step in inositol biosynthesis (Hirsch 1986). Regulation of *INO1* has been characterized in depth as it represents the most highly regulated member of the family of genes under the control of the UASino regulatory sequence, a common regulatory element in the promoter region of many lipid metabolism genes (Chang 2002**)**. Under conditions of adequate extracellular inositol, *INO1* is inactive as repressor Opi1 binds to the promoter. In the absence of extracellular inositol, derepression of *INO1* occurs as repressor Opi1 dissociates from the transcriptional activators Ino2 and Ino4 to initiate de novo synthesis of inositol (Fig. 4B and Lopes 1991).

Regulation of Sphingolipid Synthesis

The recent discovery of the ORMDL family of resident endoplasmic reticulum (ER) integral membrane proteins represents a major advancement in understanding the manner by which sphingolipid synthesis is regulated (Han 2010 and Breslow 2010). The ORMDL family is highly conserved throughout all Eukarya; in yeast, the ORMDL family members are Orm1 and Orm2 (Hjelmqvist 2002). Together, Orm1 and Orm2 negatively regulate the first, and rate-limiting reaction in sphingolipid synthesis catalyzed by SPT (see Fig. 1 and Han 2010, Breslow 2010).

Due to their negative regulatory role in SPT activity, increases in Orm protein level cause a reduction in sphingolipid synthesis (Liu 2012). One major identified condition that leads to increases in Orm2 protein is ER stress (Han 2010). ER

stress occurs in eukaryotic cells as unfolded proteins accumulate in the ER lumen (Welihinda 1999). Cells respond to ER stress by up-regulating chaperones in order to increase protein folding capacity and alleviate the accumulation of unfolded protein aggregates (Welihinda 1999). In yeast, the unfolded protein response (UPR) is activated when the luminal sensor Ire1 detects the accumulation of aggregating proteins. Activated Ire1 then splices the mRNA of transcription factor Hac1 via its cytoplasmic domain, allowing for efficient translation of Hac1 and subsequent activation of chaperones and other target genes in the nucleus (Welihana 1999).

Recently, ER stress has been shown to induce a large increase in *ORM2* transcription (see above). Though some transcription is induced through the UPR pathway as described, the bulk of the transcriptional up-regulation is dependent on calcium signaling as opposed to classic UPR induced by Ire1 activation and spliced Hac1 transcription factor (Gururaj et al, submitted manuscript). The calcium pathway responsible for increases in Orm2 protein is triggered by calcium influx through membrane channel Cch1 and activation of the calcium-dependent phosphatase, calcineurin. (Fig. 2 and Gururaj et al., submitted manuscript and Cyert 2003). Calcineurin dephosphorylates the transcription factor Crz1, allowing for its relocalization to the nucleus, and subsequent transcriptional activation of target genes (Fig. 2).

Surprisingly, cell wall stress has also been shown to induce the UPR in yeast (Scrimale 2009). Cell wall stress in yeast occurs as cell wall integrity is perturbed by several factors, including osmotic stress and elevated temperature (Jung 2002). Cell wall stress can be simulated pharmacologically with the chitin-binding reagent calcoflour white (Ram 1994). The kinase Mpk1 is primarily responsible for signaling cell wall stress and activating the cell wall integrity pathway (Levin 2005).

In addition to changes in protein level, both Orm1 and Orm2 can undergo further regulation via phosphorylation by the Npr1 and Ypk1 kinases downstream of Torc1 and Torc2, respectively (Fig. 2 and Liu 2012). Torc1 and Torc2, analogous to mTOR complexes in mammals, integrate an array of environmental cues both internally and externally in order to regulate growth and the cell cycle (Martin 2005). The Tor pathway is notably responsible for regulating the Orms in feedback response to levels of downstream products in the sphingolipid synthesis pathway (Gururaj et al., submitted manuscript). Phosphorylation of both Orm proteins via the Torc1 and Torc2 pathways results in inhibition of their negative regulatory capacity, leading to an overall increase in sphingolipid synthesis (Liu 2012, Roelants 2011, Sun 2012, Shimobayashi 2013). Although phosphorylation appears to be the major regulatory mechanism in feedback response to levels of downstream products of SPT (such as PHS and DHS), reduction in sphingolipid synthesis in response to ER stress is primarily driven by increased Orm2 protein level via the calcium signaling pathway (Gururaj et al., submitted manuscript).

In this thesis, I show that *ORM2* transcription is up-regulated in a calcium, calcineurin-dependent manner when the nutrient inositol is not available in the extracellular medium. This regulation is dependent on internal levels of inositol as I observed through manipulation of *INO1* copy number and regulatory factors Ino2, Ino4 and Opi1. And because of a previously described link between UPR and *ORM2* transcription (Han 2010 and Liu 2012), a possibility of *ORM2* up-regulation in response to cell wall stress was investigated. Shown here, Orm2 protein level increases moderately when subjected to cell wall stress induced by addition of the chitin-binding reagent calcoflour white. Together with inositol removal, these data represent two newly identified stress conditions to which cells respond by reducing sphingolipid synthesis.

Figure 1

De Novo Sphingolipid Synthesis Pathway

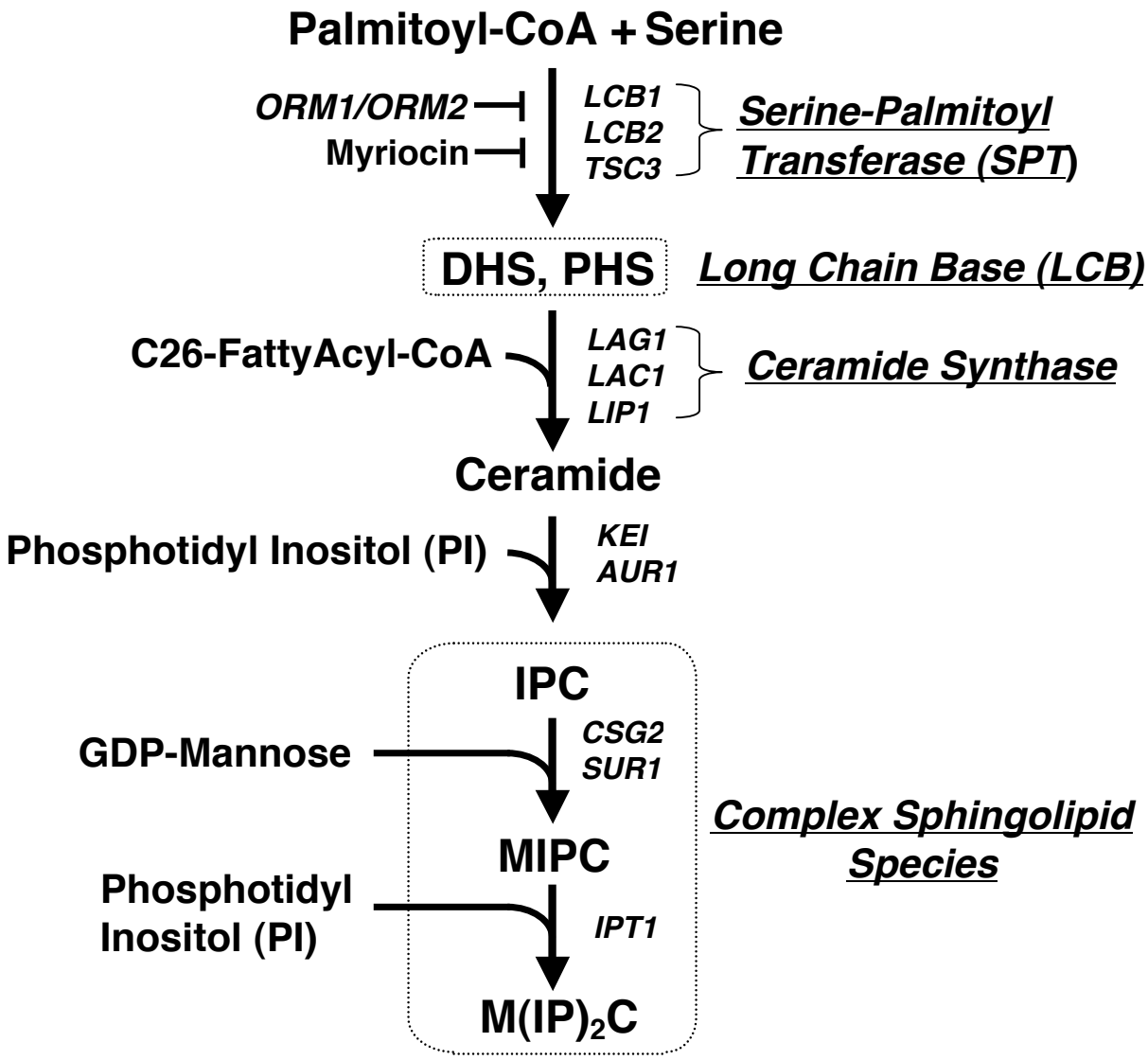
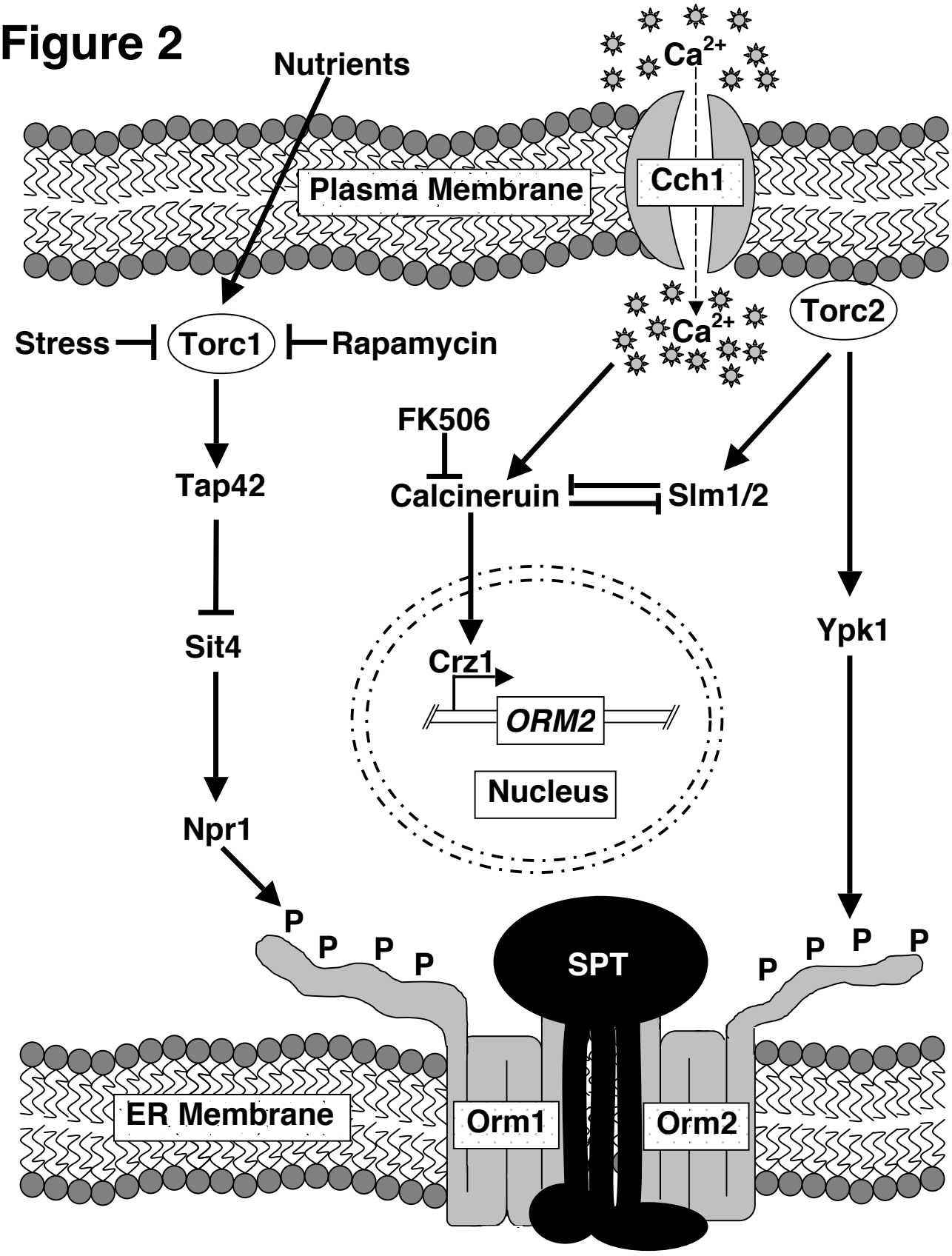


Fig. 1 - Overview of the De Novo Sphingolipid Synthesis Pathway in Yeast

Serine-Palmitoyl Transferase (SPT) (*LCB1/LCB2/TSC3*) catalyzes the first and rate-limiting reaction in sphingolipid synthesis. *ORM1* and *ORM2* negatively regulate SPT. The drug, myriocin is a specific inhibitor of SPT activity. Direct products dihyrdophingosine (DHS) and phytosphingosine (PHS), together known as long chain base, are added to C26-fattyacyl-CoA to form ceramide in the reaction catalyzed by ceramide synthase (*LAG1/LAC1/LIP1*). The complex sphingolipid IPC is formed as the *KEI/AUR1* complex incorporates phosphatidylinositol (PI) as a head group onto ceramide. Mannosylation by *CSG2/SUR1* followed by further addition of a second PI by *IPT1* creates the second and third complex sphingolipids MIPC and M(IP)2C respectively

Simplified from Funato 2002

Figure 2



Modified from De Virgillo 2006, Loewith 2011, Liu 2012, Cui 2009, Bultynck 2006 and Han 2004

Fig. 2 (previous page) - Orm proteins modulate their regulation of sphingolipid synthesis via protein level changes induced through calcium signaling, and changes in phosphorylation state through Torc1 and Torc2 signaling networks

Orm1 and Orm2 associate in a complex with serine-palmitoyl transferase (SPT) in the ER membrane. Orm2 transcription is activated via calcium signaling: influx of calcium through membrane channel Cch1 activates calcineurin which deposphorylates the transcription factor Crz1, allowing for its entry into the nucleus where it activates transcription of *ORM2*. Calcineurin is inhibited by the drug FK506, and can be negatively regulated by Slm1/2 downstreatm of Torc2. Torc1 and Torc2 integrate cellular cues and regulate a wide array of cell cycle and growth pathways. Torc1 is activated by nutrient availability and inhibited by stress conditions; Torc1 is inhibited by the drug rapamycin. Active Torc1 leads to inhibiton of Sit4 and any further signaling to Npr1 is repressed. When Torc1 is inhibited, the serine-threonine phosphatase Sit4 is activated, leading to activation of the kinase Npr1. Orm1 phosphorylation is dependent on Npr1 (Liu et al 2012). Phorsphorylation of Orm1 reduces its activity as a negative regulator of SPT, and thus an overall increase in sphinoglipid synthesis occurs. In parallel with Torc1, Torc2, when *active*, stimulates the kinase Ypk1, which stimulates Orm2 phosphrylation. Orm2 phosphorylation relieves repression of SPT activity, leading to an overall increase in sphingolipid synthesis. Simultaneously, active Torc2 stimulates Slm1/2 which inhibit calcineurin and reduce Orm2 transcription.

Experimental Procedures

Strains and Media

Yeast strains are isogenic with BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Strains from the deletion collection (Open Biosystems, Huntsville, AL) were confirmed by PCR. For Orm2-TAP studies, strains were made by mating ACX184-2B [MATa *ORM2::TAP::HIS3* (Open Biosystems)] with MATa deletion strains as follows: ACX204-3B is *cnb1Δ::kan*^{r}, ACX230-3B is *ino2Δ::kan*^{r}, ACX231-2B is *ino4Δ::kan*^{r}, ACX234-1C is *cch1Δ::kan*^{r}, ACX275-4B is *ino1Δ::kan*^{r}.

Unless otherwise noted, experiments were carried out in standard synthetic complete (SC) yeast media (Sherman 1986). For cells harboring plasmids with selectable amino acid markers, cells were grown in SC medium lacking the corresponding amino acid. For inositol starvation experiments, cells were grown to mid log phase in SC, pelleted, washed with water, and resuspended in SC minus inositol media, or SC minus inositol and corresponding amino acid for cells harboring a plasmids. Cell wall stress assays were carried out by growing cells to mid log phase in SC, back diluting, and growing for six additional hours in YPD media before incubation with calcoflour white as the reagent is non-functional in SC (Krysan 2005).

Plasmids

An *MPK1-lacZ* reporter construct (p1365), as described in (Jung 2002), was from David Levin (Johns Hopkins University, MD). The UPRE-*lacZ* reporter (pJC106), as described in (Cox 1993), was from Peter Walter (UCSF), the *INO1-lacZ* reporter (pJH359), as described in (Lopes 1991), was from Susan Henry (Cornell University, NY) and the 2μ*INO1* (pJH318) as described in (Hirsch 1986) was from Jana Patton-Vogt (Duquesne University, PA).

Electrophoretic mobility shift assay, Western blot, and enzyme assay

For quantitative Western blots, lysate was prepared by vortexing with glass beads in sorbitol buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris, pH 7.4) with a protease inhibitor cocktail and phenylmethylsulfonyl fluoride, as described previously (Chang 1991). To assay Orm phosphorylation, cells were harvested and frozen in liquid nitrogen and trichloroacetic acid, as described (Liu 2012). Orm2-TAP mobility shifts were visualized after extended electrophoresis on 12% polyacrylamide gels. Reduced mobility of Orm2-TAP reflects increased phosphorylation as alkaline phosphatase treatment collapsed slower migrating bands to a single fast migrating band (Liu 2012). For phosphorylation analysis, lysate was prepared by vortexing with glass beads in the presence of trichloroacetic acid. Western blots were visualized with rabbit antibody (to detect the TAP tag) followed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection. ImageJ software was used for quantitation of bands. For *lacZ* (*UPRE-lacZ*, *MPK1-lacZ*, and *INO1-lacZ*) assays, lysate was prepared in breaking buffer (20% glycerol, 1 mM DTT, 100 mM Tris, pH 8) or bead buffer with protease inhibitors, and ß galactosidase activity was measured as described previously (Rose 1990). Samples were normalized to protein content assayed by Bradford or BCA (Pierce) assays.

Results

Orm2 Protein Increases in Response to Loss of Inositol from the Media

Because of the nature by which Orm2 regulates the rate-limiting step in sphingolipid synthesis (Han 2010 and Breslow 2010), and the requirement PI for the creation of complex sphingolipids (Funato 2002), I investigated whether cells might respond to a loss of inositol in the growth media with a change in Orm2 protein level. Orm2 protein level was analyzed using cells with a chromosomal *ORM2* tagged with a TAP epitope tag. At various times after shifting cells to inosiol-free media, Orm2-TAP level was quantitated by Western blot. Figure 3B shows that Orm2 protein level increases about three fold, three hours after shift to inositol free media. No additional increase was seen after three hours, and this is the maximal Orm2 induction observed in response to inositol removal (Fig. 3B). This is a modest increase in comparison to the 10-11 fold increase observed in response to ER stress (elicited by addition of tunicamycin to inhibit N-linked glycosylation [(Fig. 3C) and Gururaj et al., submitted manuscript). Thus, cells respond to removal of inositol from the medium with increased Orm2 protein levels.

Yeast import extracellular inositol through two transporters, Itr1 (high affinity) and Itr2 (low affinity) (Nikawa 1991 and Miyashita 2003). To determine whether either protein is also responsible for sensing the loss of inositol in the media and signaling to induce an Orm2 increase, *itr1Δ* and *itr2Δ* mutants were shifted to inositol-free medium for 3 hours and Orm2-TAP levels assayed via Western blot. There was no detectable change in Orm2 protein level in response to inositol removal in either of the mutants relative to wild type cells (Fig. 3C). Tunicamycin addition was used as a positive control. It appears that the transporters Itr1 and Itr2 do not play a role in the Orm2 response to loss of inositol. However, it is possible that each transporter can fully compensate for the loss of the either, including the potential sensing of inositol availability. Little conclusive data is

currently available regarding the functionality of Irt1 and Itr2 proteins and the above possibility cannot be ruled out. Given this and the lack of detectable phenotypic difference in either mutant alone, I considered an alternative hypothesis that the Orm2 response is primarily driven by differences in *internal* inositol levels caused by the removal of extracellular inositol.

Orm2 Increase is Dependent on Intracellular Inositol Levels When Extracellular Inositol is Absent

Cells compensate for a loss of extracellular inositol through derepression of the gene *INO1*; the Ino1 protein is responsible for catalyzing the rate-limiting step in de novo inositol biosynthesis (White 1991). This occurs through a mechanism by which repressor Opi1 dissociates from transcription factors Ino2 and Ino4 [(Henry 2012) and Fig. 4B left panel]. An *INO1-LacZ* construct was used to detect *INO1* transcriptional induction. Wild-type cells respond to removal of inositol by inducing transcription of *INO1*, while cells lacking transcription factors Ino2 or Ino4 fail to do so (Fig. 4B right panel). Furthermore, cells lacking the repressor Opi1 show constitutively high *INO1* transcription under all conditions tested (Fig. 4B right panel).

In order to ascertain whether the increase in Orm2 occurs in response to intracellular levels of inositol when extracellular inositol is removed, *ino2Δ, ino4Δ, and opi1Δ* mutants were subjected to a three-hour incubation in inositol-free media and Orm2-TAP levels assayed via Western blot. In contrast to wild-type cells, Orm2 does *not* increase in *opi1Δ* mutants after a shift to inositol-free medium (Fig. 4A). Similarly, Orm2 does not increase in cells harboring a high copy 2μ plasmid containing *INO1* (Fig. 4C). In both *opi1Δ* and *INO1* over-expressing cells, internal inositol levels remain high due to increased *INO1* activity. By contrast, *ino2Δ* and *ino4Δ* cells cannot de-repress the *INO1* gene, and show constitutively increased Orm2 protein, even in the presence of extracellular inositol. Orm2 protein level is further increased when *ino2Δ* and

ino4Δ cells are starved for inositol (Fig. 4A). Finally, deletion of *INO1* itself caused no change when extracellular inositol was present but showed maximal Orm2 transcription when shifted to inositol-free media (Fig. 4C compare –ino and +tun for wild-type and *Ino1Δ cells*). These data suggest that Orm2 protein level increases in response to the level of intracellular inositol when extracellular inositol is absent.

ORM2 Transcription in response to Loss of Inositol From the Media is Signaled via a Calcium, Calcineurin-Dependent Pathway

The detailed mechanisms behind regulation of Orm protein levels have remained mysterious. However, a recent and important finding is that Orm2 increases in response to ER stress (induced by tunicamycin or the reducing agent DTT) are signaled via a calcium and calcineurin-dependent pathway (Gururaj et al., submitted manuscript). Figures 2 and 5B illustrate the calcium-signaling pathway. An influx of calcium through membrane channel Cch1 activates the phosphatase calcineurin, which in turn activates the transcription factor Crz1, leading to an increase in transcription of *ORM2* (Cyert 2003 and Gururaj et al., submitted manuscript). Because this pathway has been identified as playing a major role in *ORM2* transcription from ER stress, I asked whether calcium, calcineurin-dependent signaling is also involved in response to removal of inositol. Both pharmacological, and genetic approaches were taken to perturb the pathway's activity. The immunosuppressant FK506 has been shown to be a potent inhibitor of calcineurin (Fig. 2, 5B, and Clipstone 1992). To confirm the efficacy of FK506 on calcineurin signaling, tunicamycin was added with or without various concentrations of FK506 and Orm2 protein level was determined via Western blot (Fig. 5A). As shown, all three concentrations of FK506 tested abrogated the response to ER stress brought on by tunicamycin addition. FK506 was then added to cells at the lowest dose during the full three hours of incubation in inositol-free media. As shown in Fig. 5B (left panel), Orm2 response was diminished under these conditions. Similarly, a decrease in Orm2

response to inositol-free medium was observed in *cnb1Δ* mutants lacking the regulatory subunit of calcineurin (Fig. 5B right panel), as well as in *cch1Δ* mutants lacking the membrane calcium channel (Fig. 5C). A summary of relative increases in Orm2 protein levels quantified from three independent experiments confirms the role of the calcium, calcineurin pathway in Orm2 transcription in response to loss of inositol in the medium (Fig. 5C).

Phosphorylation of Orm2 is Unaffected by Loss of Inositol From the Media

In addition to changes in Orm2 protein level, sphingolipid synthesis is regulated by phosphorylation of both Orm1 and Orm2 through the Torc1 and Torc2 signaling pathways (Liu 2012, Roelants 2011, Sun 2012, Shimobayashi 2013). Both Orm proteins are inhibited by phosphorylation, resulting in decreased negative regulation of SPT and a net increase in sphingolipid synthesis (Fig. 2). To test whether sphingolipid synthesis responds to inositol removal via phosphorylation of Orm2, I used Orm2 electrophoretic mobility shift on SDS-PAGE gels to assay phosphorylation (Fig. 6A). Other than an increase in protein level (similar to what was reported above), no changes were seen in phosphorylation patterns after a three-hour shift to inositol-free media. This is in stark contrast to the positive control: addition of the SPT inhibitor myriocin, which inhibits SPT and resulted in maximal phosphorylation of Orm2 (Fig. 6A). In *opi1Δ* mutants, no changes were seen in Orm2 phosphorylation after three hours in inositol-free media; the increase in protein level observed in WT strains upon inositol removal was absent (Fig. 6A), *consistent* with Fig. 4 above.

UPR Induced by Inositol Removal is Independent of Signaling to Increase Orm2 Protein

Removal of inositol from the media has been shown to induce the unfolded protein response in yeast, although the reason is not well understood (Promlek 2011 and Henry 2012). Because ER stress causes both UPR induction and

increased Orm2 protein level, I investigated the connection between UPR, as triggered by loss of inositol, and increases in Orm2 protein. Because this increase in Orm2 is dependent on calcium signaling (Gururaj et al., submitted manuscript), I sought to determine whether calcium signaling is also responsible for inducing UPR in the absence of extracellular inositol. UPR induction was assayed via an *UPRE-LacZ* construct in cells lacking calcium signaling components *CNB1* and *CCH1*, as well as with the addition of FK506. Unlike Orm2 protein increase which relies on the calcium signaling pathway, UPR activity is unaffected by the inhibition of this pathway by FK506 or in *cnb1Δ* and *cch1Δ* mutants (Fig. 6B).

Cell Wall Stress Triggers an Increase in Orm2 Protein via Mpk1

In addition to ER stress, cell wall stress has also been shown to induce the UPR (Scrimale 2009). To determine whether Orm2 increases in response to cell wall stress, the chitin-binding reagent calcoflour white (Ram 1994) was added to cells. Orm2 protein is slightly increased in wild-type cells upon addition of calcoflour white (Figs. 7A-B). Mpk1, one of the MAP kinases, is responsible for signaling cell wall stress (Levin 2005). In *mpk1Δ* mutants, Orm2 response to calcoflour white was abrogated, confirming that Orm2 increase in response to cell wall stress is signaled through Mpk1 and the cell wall integrity response pathway (Fig. 7A). By contrast, Orm2 response to ER stress-inducing agents DTT and tunicamycin remained unaffected in *mpk1Δ* cells (Fig. 7A).

In order to determine whether calcium signaling is involved in Orm2 transcription under cell wall stress conditions, Orm2 level in *cnb1Δ* mutants was assayed after calcoflour white addition. Similar to ER stress and inositol removal, Orm2 response to cell wall stress was abrogated in *cnb1Δ* cells (Fig. 7A). To ascertain whether Orm2 response to tunicamycin (as tunicamycin may itself induce cell wall stress) and cell wall stress induced by calcoflour white are independent, an

MPK1-LacZ construct was used to measure cell wall stress activation (Jung 2002). Maximal *MPK1* induction was observed in cells treated with calcoflour white, while only minimal activity was observed in cells treated with tunicamycin (Fig. 7B). Furthermore, large increases in Orm2 were seen after tunicamycin addition in the absence of *MPK1,* while deletion of *MPK1* abrogated the Orm2 response to calcoflour white (Fig. 7A compare *mpk1Δ* +T, +D, and +C). Thus, cell wall and ER stress signal to increase Orm2 protein via calcineurin, but from two different pathways.

Figure 7C shows a model whereby ER stress, removal of inositol, and cell wall stress signaling via Mpk1 all work through calcineurin to increase transcription of *ORM2* to down-regulate sphingolipid synthesis.

Figure 3

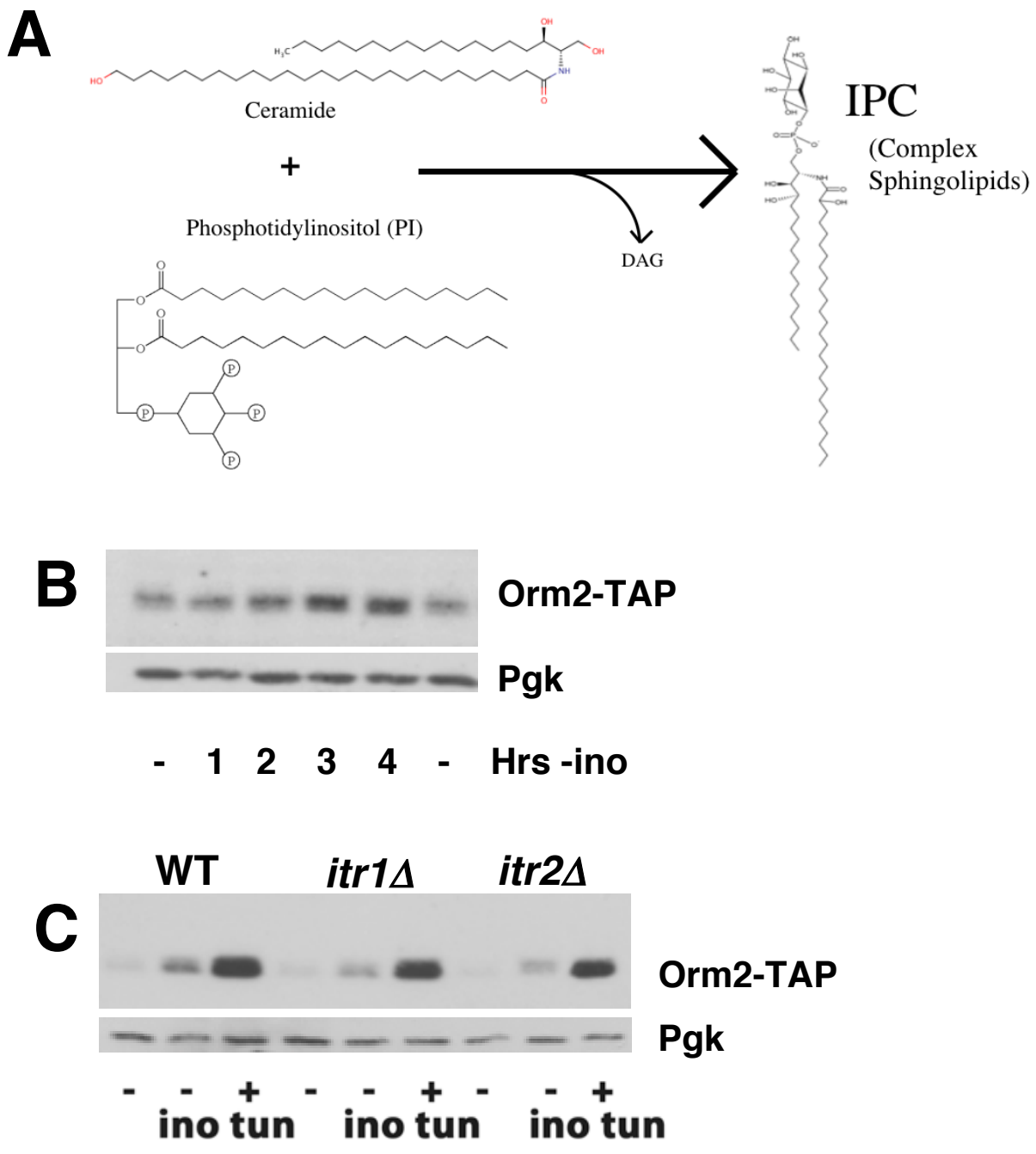


Fig. 3 - Orm2 increases when inositol is removed from the media

(A) Phosphotidylinositol (PI) is conjugated onto ceramide as the head group in the complex sphingolipid species. (B) Western blot of Orm2-TAP in cells shifted from SC medium to inositol free medium for 4h at 30°C with time points taken prior to the shift, and at each hour after. Maximal Orm2 induction in response to inositol loss occurs 3h after the media shift. In B & C, Pgk1 is the loading control. (C) Signaling the inositol loss to induce Orm2 does not require inositol transporters Itr1 or Itr2. Addition of tunicamycin (1μg/ml) for 2h was used as a positive control, and induces maximal transcription of Orm2.

Figure 4

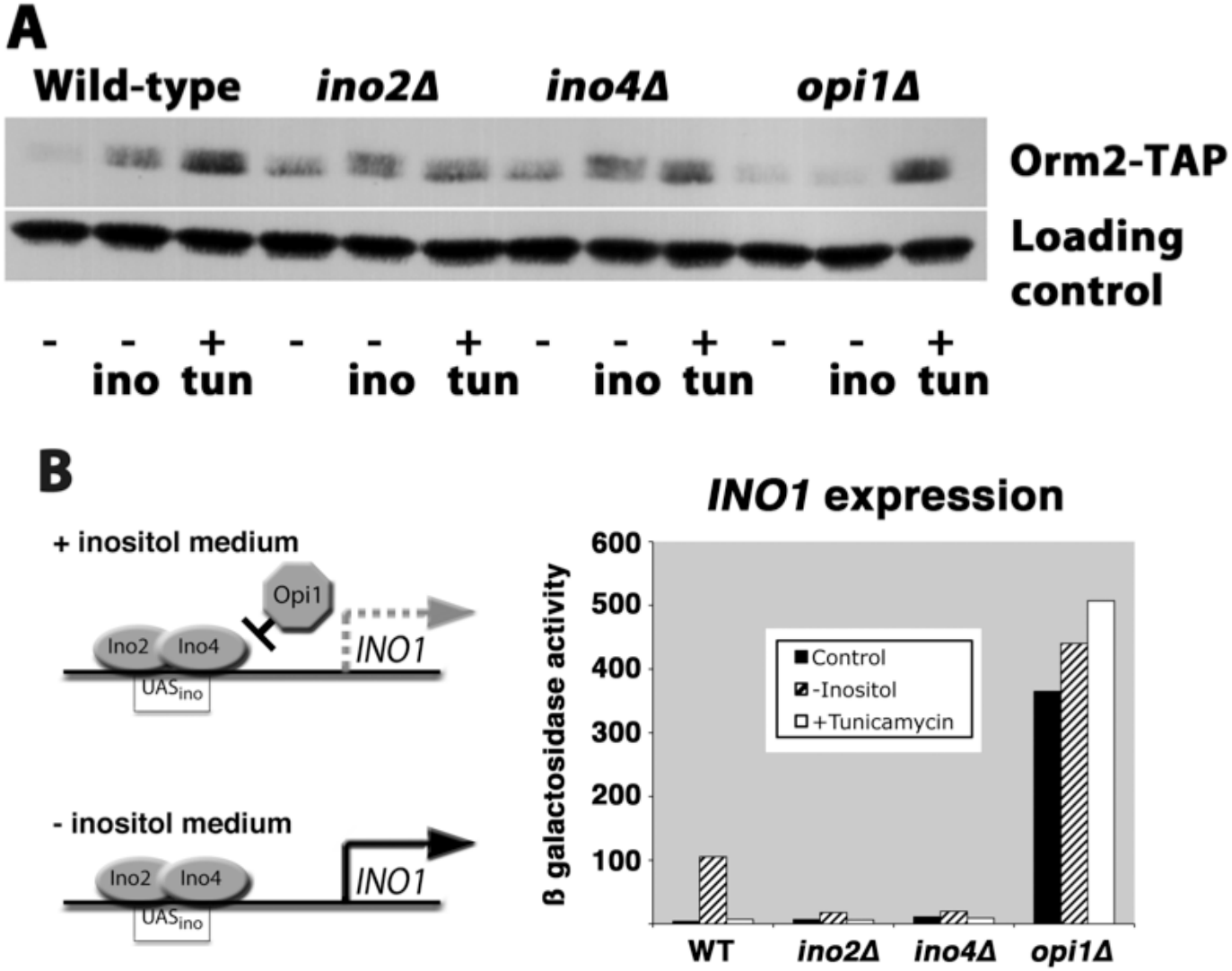


Fig. 4 - Orm2 level responds to internal insoitol levels when no longer available in the media

(A) Western blot measurement of Orm2-TAP levels in cells incubated in SC medium, inositol-free medium for 3h, or tunicamycin (tun, 1 µg/ml) for 2h at 30°C. Increased Orm2 in response to inositol-free medium is prevented by *opi1Δ;* Orm2 is constitutively increased in *ino2Δ* and *ino4Δ* cells. (B) *Left panel*, schematic of *INO1* transcriptional regulation. *Right panel*, derepression of *INO1* in cells incubated in inositol-free medium as measured by an *INO1-lacZ* reporter. ß-galactosidase activity in cell lysate is expressed as µmol/min/mg. (C) Western blot showing Orm2-TAP levels in *ino1Δ* cells and those harboring a 2.µ*INO1* plasmid. Pgk is the loading control.

Figure 5

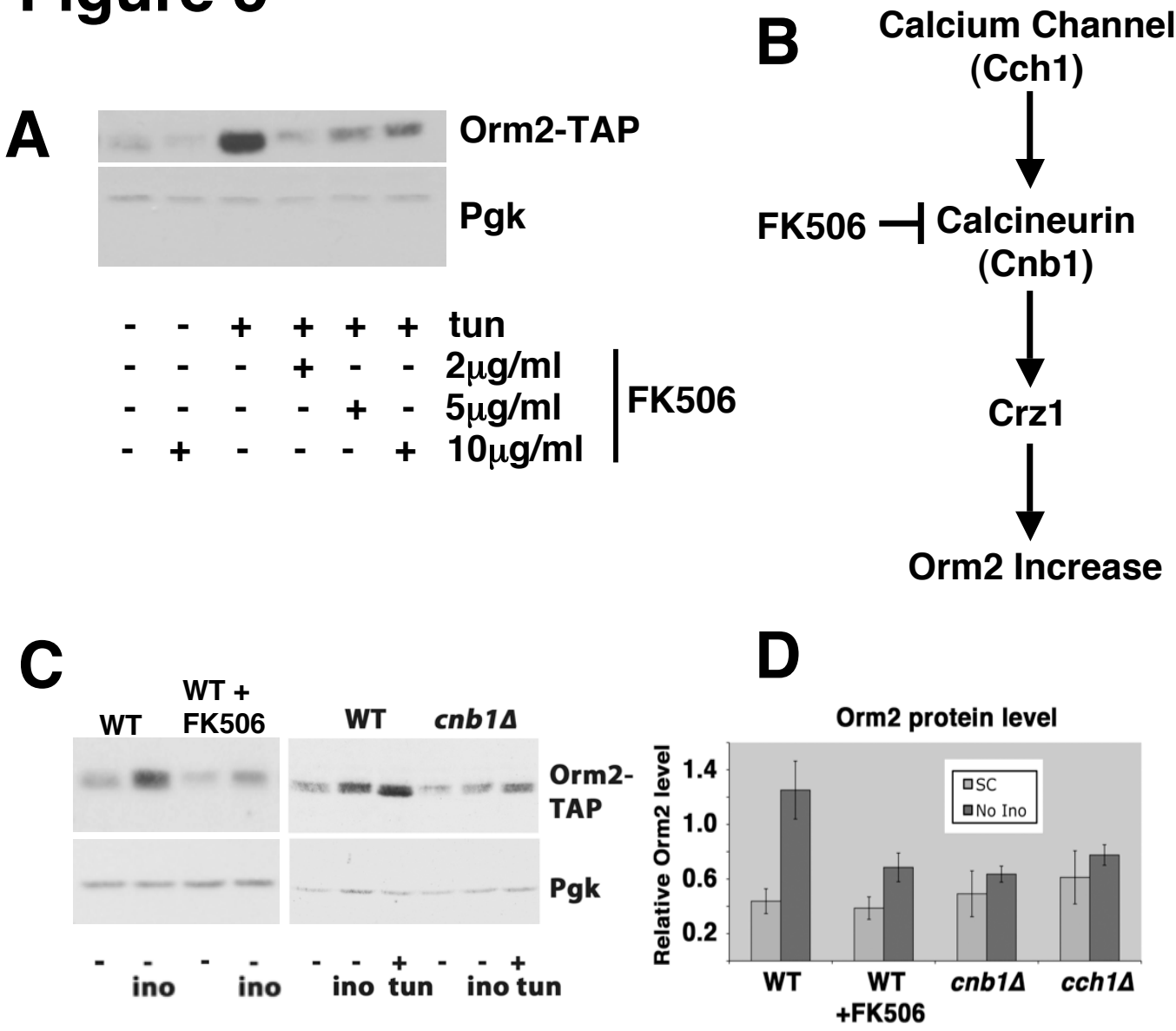


Fig. 5 - Orm2 increase in response to inositol loss is dependent on calcium signaling

(A) Western blot of Orm2-TAP response with the addition of tunicamycin alone or with potent calcineurin inhibitor FK506 in SC media for 2hrs at 30°C. In A & C, Pgk was assayed as a loading control. (B) Simplified diagram of the calcium signaling pathway responsible for activating transcription of *ORM2*. (C) Orm2-TAP response to inositol starvation is abrogated when FK506 is added at 2 μg/ml during the three-hour starvation, and in *cnb1Δ* mutants. (D) Quantitation of Orm2-TAP response to inositol-free medium of wild-type cells, wild-type cells treated with FK506 (2 μg/ml for 3h), *cnb1*Δ cells, and *cch1*Δ cells. Error bars from n = 3.

Figure 6

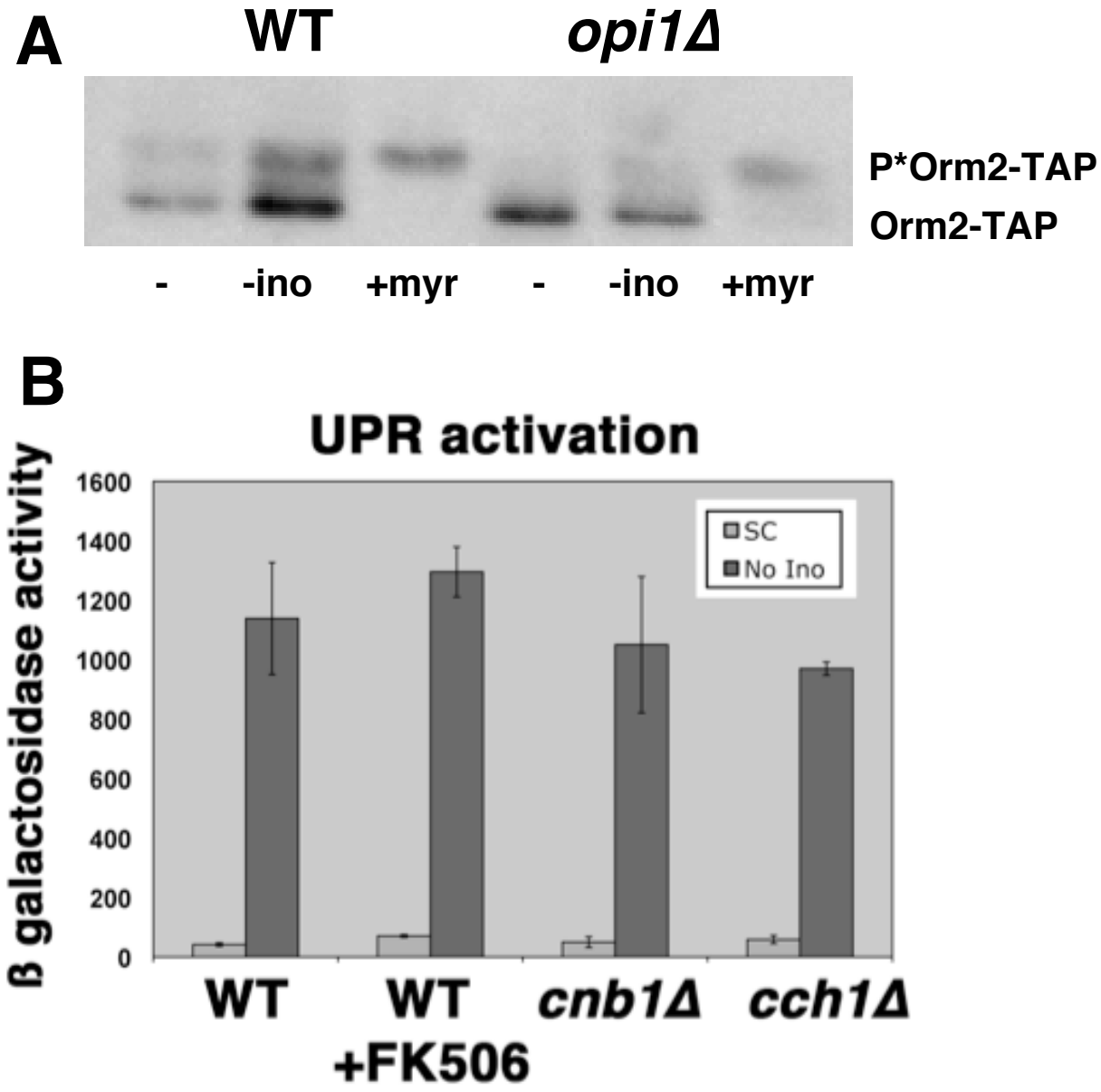


Fig. 6 - Orm2 Phosphorylation does not respond to inositol loss and UPR activation by inositol loss is not dependant on calcium signaling

(A) Mobility shift western blot showing the relative abundance of Orm2-TAP and P*Orm2-TAP in WT and *opi1*Δ cells. Addition of myriocin to cells in SC for 1hr was used as a positive control and induces maximal phorphorylation of Orm2. (B) UPR activation in inositol-free medium. Wild-type, *cnb1*Δ, and *cch1*Δ cells were shifted to inositol-free medium for 3h with out without the addition of FK506 (2 µg/ml), and UPR was assayed by a *UPRE-lacZ* reporter; ß-galactosidase activity in cell lysate is expressed as µmol/min/mg. Error bars represent n=3.

Figure 7

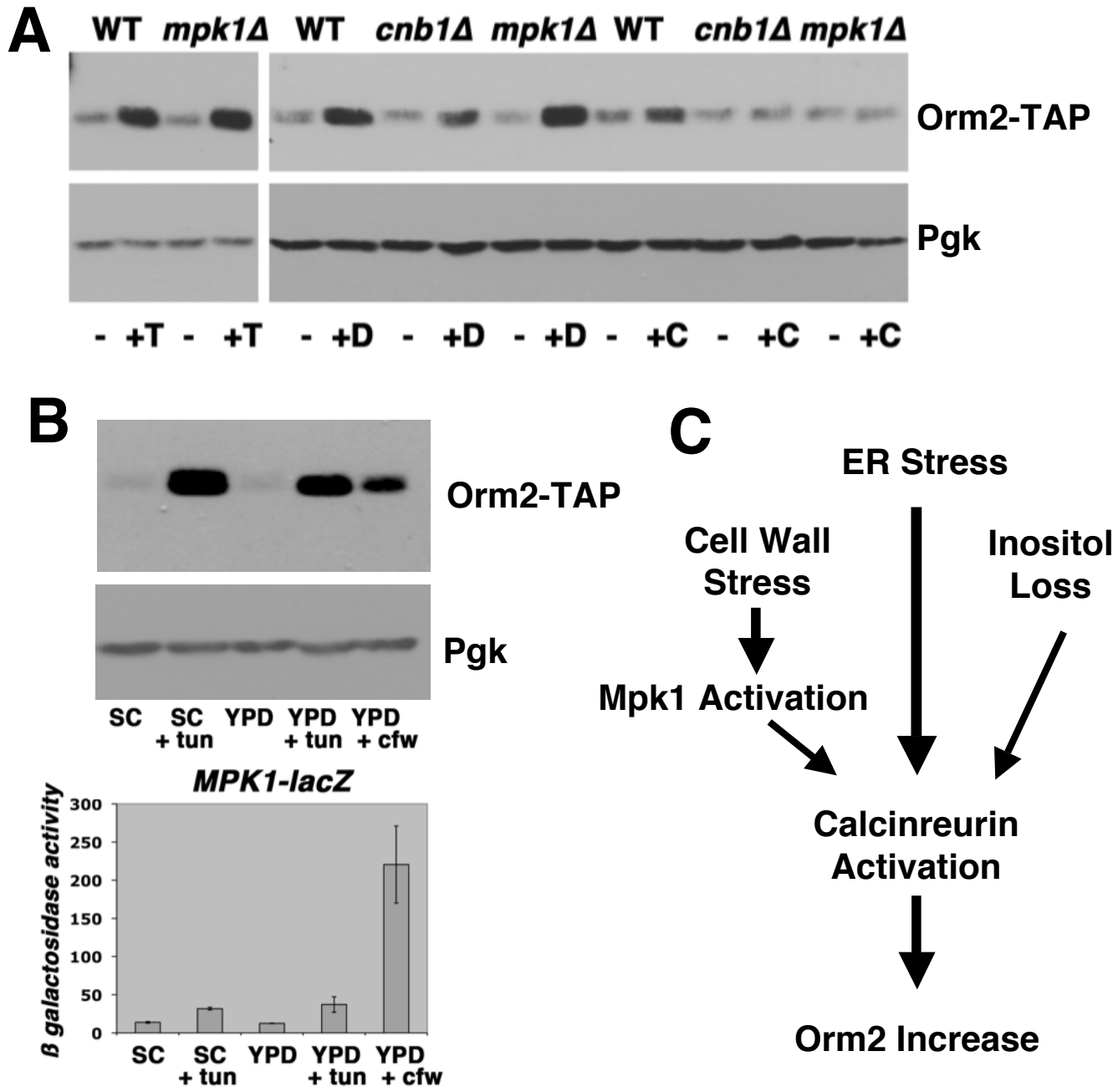


Fig. 7 Orm2 protein level is increased by calcofluor white-induced cell wall stress.

Cells were grown overnight to mid-log phase in SC-uracil medium at 30°C and then shifted to YPD for 6h before treatment with tunicamycin (1 µg/ml), DTT (1 mM) or calcofluor white (40 µg/ml) for an additional 2h. Lysates were assayed for both Orm2-TAP levels and activation of cell wall stress signaling. (A) Western blot for Orm2-TAP in cells treated +/- tunicamycin (+T), DTT (+D), or calcofluor white (+C). (B) ß galactosidase activity (µmol/min/mg) of an *MPK1-lacZ* reporter to quantitate cell wall stress signaling, and corresponding Western blot showing Orm2-TAP levels. Cell wall stress is maximally induced by calcofluor white. (C) Diagram showing ER stress-induced Orm2 increase and increase in response to inositol loss is dependent on calcineurin activation; cell wall stress-induced Orm2 increase is signaled through Mpk1 to calcineurin activation.

Discussion

Along with phosphorylation of the Orm proteins, a change in Orm2 levels represents one of the primary mechanisms by which cells maintain sphingolipid homeostasis (Han 2010 and Breslow 2010). Characterized here are two cellular stress conditions whose role in down regulating sphingolipid synthesis can be inferred through the observed increase in Orm2 protein level. Both loss of inositol from the media and cell wall stress triggered increased transcription of *ORM2.* Though the possibility of decreased degradation of Orm2 cannot be overlooked, the reduced responses to these conditions seen in calcium signaling mutants or with FK506 is consistent with increased transcription of *ORM2* as previously reported (Gururaj et al., submitted manuscript).

The highly regulated nature of the *INO1* gene allowed for a detailed approach to examining the observed Orm2 increase when inositol was removed from the media. Induction of Orm2 protein by shifting cells to inositol-free media is independent of *INO1*-regulated de novo inositol synthesis and the factors responsible for derepression of *INO1,* as the Orm2 increase still occurs in *ino1*Δ*,* *ino2*Δ, and *ino4*Δ cells. Instead, calcium signaling, responsible for *ORM2* transcriptional activation induced by ER stress (Gururaj et al., submitted manuscript), is also the key pathway in signaling Orm2 increases when intracellular inositol is low (Villa-Garcia 2011). With the exception of *ino2Δ* and *ino4Δ* mutants, all strains tested showed wild type levels of Orm2 when extracellular inositol is present. The extent to which Orm2 increases after the shift to inositol-free media appears to be dependent on the intracellular levels of inositol in the absence of extracellular inositol. No increase in Orm2 was observed in either *opi1Δ* mutants, or cells over-expressing *INO1* (on a 2μ plasmid). This is likely due to over-expression of the *INO1* gene product leading to constitutively high levels of inositol within the cell under all conditions. Consistently, *ino1Δ* mutants showed a much stronger Orm2 response relative to wild-type when shifted to inositol-free media, reflecting the fact that *ino1Δ* cells

cannot produce any new inositol when it is no longer available from the media (White 1991), resulting in a greater loss of intracellular inositol.

A unique phenotype was seen with *ino2Δ* and *ino4Δ* mutants. These strains were the only cells tested that showed constitutively increased Orm2 protein, even in the presence of extracellular inositol (Fig. 4). This is consistent with reports suggesting that *ORM2* is under negative regulation by the transcription factors Ino2 and Ino4 (Santiago 2003); and thus their absence would lead to the observed increases in *ORM2* transcription regardless of the availability of inositol. With the calcium signaling network intact, the further increase of Orm2 upon inositol removal is best explained by additional positive transcriptional regulation in a calcineurin dependent manner.

A second major finding of this work is that cell wall stress, induced by calcoflour white, leads to an increase in Orm2 protein level (Fig. 7). Using the *MPK1-LacZ* construct as an assay for cell wall stress response, it was confirmed that the calcoflour white-induced increase in Orm2 protein was independent of ER stress, as tunicamycin addition increases Mpk1 activity minimally relative to calcoflour white. Furthermore, deletion of *MPK1* abrogated the Orm2 increase in response to cell wall stress but had no effect on the response to either tunicamycin or DTT.

Signaling via calcineurin effects plasma membrane remodeling in response to a variety of stress signals (Cyert 2003). In addition to demonstrating a role for calcium signaling in modifying lipid metabolism in response to ER stress, here a larger role for the calcium, calcineurin pathway can be seen. It would appear that calcium signaling is fueled by several stress sensing systems in order to adjust sphingolipid synthesis in response to these stressors and to modify the makeup of cell membranes accordingly to compensate.

Though major advancements have recently been made in understanding the roles that sphingolipids and their metabolites play in membrane structure and

function, the details of these interactions are still poorly understood. The discovery of membrane microdomains marked a significant change in the way in which the dynamics of lipids and membrane-bound proteins were thought to behave. As sphingolipids are key components of microdomains and play a major role in regulating the overall dynamics of the plasma membrane, understanding their regulation is paramount in identifying how cells maintain their lipid homeostasis under trying conditions. My current work in progress is focused on examining how turnover and stability of integral membrane proteins is affected by changes in sphingolipid content of the plasma membrane and through signaling activated by sphingolipid metabolites. Efforts to elucidate trafficking and turnover of membrane proteins have been focused primarily on signaling and vesicle regulation. It is becoming increasingly clear that sphingolipids play a major role in coordinating the lipid environment and the protein content of the plasma membrane through simultaneous lipid content adjustments and signaling via metabolites to protein trafficking systems. Sphingolipid regulation is a critical part of understanding how the lipid environment and lipid metabolites contribute to regulating these membrane-based processes so crucial to the life and growth of the eukaryotic cell.

Finally, It should be noted that the majority of this work is included in the most recent manuscript submitted for publication from the Chang Lab: Orm proteins integrate multiple signals to maintain sphingolipid homeostasis, Charulatha Gururaj, Ross Federman, and Amy Chang. Furthermore, my contribution to the above manuscript for publication extends beyond the scope of this work including experiments and data not found herein.

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