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Orm Proteins Integrate Multiple Signals to Maintain Sphingolipid Homeostasis*

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Background: Orm proteins are key regulators of sphingolipid synthesis.
Results: Orm1 and Orm2 are phosphorylated by TORC1 and TORC2; Orm2 is transcriptionally regulated by a calcium- and calcineurin-dependent pathway.
Conclusion: The Orm proteins respond to feedback signals from the sphingolipid synthesis pathway and multiple environmental signals.
Significance: Modulation of sphingolipid synthesis is a critical component of cellular response to nutritional status and stress.

Sphingolipids are structural components of membranes, and sphingolipid metabolites serve as signaling molecules. The first and rate-limiting step in sphingolipid synthesis is catalyzed by serine palmitoyltransferase (SPT). The recently discovered SPT-associated proteins, Orm1 and Orm2, are critical regulators of sphingolipids. Orm protein phosphorylation mediating feedback regulation of SPT activity occurs in response to multiple sphingolipid intermediates, including long chain base and complex sphingolipids. Both branches of the TOR signaling network, TORC1 and TORC2, participate in regulating sphingolipid synthesis via Orm phosphorylation in response to sphingolipid intermediates as well as nutritional conditions. Moreover, sphingolipid synthesis is regulated in response to endoplasmic reticulum (ER) stress by activation of a calcium- and calcineurin-dependent pathway via transcriptional induction of *ORM2*. Conversely, the calcium- and calcineurin-dependent pathway signals ER stress response upon lipid dysregulation in the absence of the Orm proteins to restore ER homeostasis.

Sphingolipids are structural components of cell membranes, regulating membrane fluidity. On the outer leaflet of the membrane, sphingolipids assemble laterally with cholesterol to form membrane microdomains that provide conformational support for membrane proteins and serve as platforms for recruitment of signaling molecules (1). In addition to a structural role, sphingolipids have critical regulatory activities; sphingolipid precursors/metabolites, such as ceramide, participate as second messengers in signaling to protein kinases and phosphatases. Like phosphoinositides, which act as spatial determinants of membrane trafficking events, sphingolipid metabolites play an important role in actin cytoskeleton polarization and endocytosis (2).

Discovery of the ORMDL family of endoplasmic reticulum (ER)[2] membrane proteins represents a major advance in understanding regulation of sphingolipid synthesis (3, 4). The physiologic importance of the ORMDL family is underscored by the finding that one of the human family members is an asthma susceptibility gene (5). In yeast, the ORMDL family members Orm1 and Orm2 are negative regulators of the first and rate-limiting step in sphingolipid synthesis, a reaction catalyzed by serine palmitoyltransferase (SPT). Orm1 and Orm2 proteins regulate SPT activity by physically associating with it (3, 4). Recent evidence suggests that TORC1 and TORC2 protein kinase complexes adjust sphingolipid synthesis via phosphorylation of the Orm proteins as they coordinate cell growth with environmental changes, such as nutrient availability or plasma membrane stress (6–9).

We now show that sphingolipid synthesis is regulated via Orm phosphorylation by a feedback mechanism that responds to multiple sphingolipid intermediates, including long chain base as well as complex sphingolipid(s). In response to ER stress, sphingolipid synthesis is modulated by a calcium- and calcineurin-dependent pathway that regulates Orm2 protein level. Increased Orm2 protein has been shown to increase repression of SPT activity to inhibit sphingolipid synthesis (6). Conversely, lipid dysregulation in the absence of the Orm proteins activates calcium- and calcineurin-dependent signaling to ameliorate ER stress.

EXPERIMENTAL PROCEDURES

Strains and Media—Standard yeast media and genetic manipulations were as described (10). Yeast strains are isogenic with BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Orm mutants were *orm1*Δ::*clonNAT*^{r} (HXX1-7B), *orm2*Δ::*kan*^{r} (HXX1-7A), and *orm1*Δ *orm2*Δ (HXX1-7D), as described previously (3). Strains from the deletion collection (Open Biosystems, Huntsville, AL) were confirmed by PCR. For Orm2-TAP studies, strains were made by mating ACX184-2B (MATα *ORM2*::*TAP*::*HIS3* (Open Bio-

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[2] The abbreviations used are: ER, endoplasmic reticulum; SPT, serine palmitoyltransferase; UPR, unfolded protein response; PHS, phytosphingosine; AbA, aureobasidin A; TAP, tandem affinity purification.

systems)) with MATa deletion strains as follows. ACX195-2D is *ire1*Δ::*kan*^{r}, ACX201-5C is *crz1*Δ::*kan*^{r}, ACX204-3B is *cnb1*Δ::*kan*^{r}, ACX230-3B is *ino2*Δ::*kan*^{r}, ACX231-2B is *ino4*Δ::*kan*^{r}, ACX234-1C is *cch1*Δ::*kan*^{r}, ACX245-1D is *hac1*Δ::*kan*^{r}, ACX254-2B is *csg2*Δ::*kan*^{r}, ACX261-3C is *elo3*Δ::*kan*^{r}, ACX265-3D is *isc1*Δ::*kan*^{r}, ACX268-4A is *ire1*Δ::*kan*^{r} *cnb1*Δ::*kan*^{r}, ACX275-4B is *ino1*Δ::*kan*^{r}, and ACX233-1C is *ypk1*Δ::*kan*^{r}. ACX264-4C is from a cross between ACX184-2B and KSY271 (*kei1*Δ::*kei1-1*::*LEU2*; a gift from Koji Yoda (University of Tokyo)) (11).

Plasmids—pSH14HA is a *HIS3*-marked centromeric plasmid bearing *ORM1* tagged at the amino terminus with an HA epitope, as described (6). An *MPK1-lacZ* reporter construct (p1365), as described previously (12), was from David Levin (The Johns Hopkins University, Baltimore, MD). The UPRE-lacZ reporter (pJC106), as described previously (13), was from Peter Walter (University of California, San Francisco), and the *INO1-lacZ* reporter (pJH359), as described previously (14), was from Susan Henry (Cornell University).

Semiquantitative PCR—RNA was isolated using RNeasy (Qiagen) or Ribopure Yeast (Ambion) kits, according to the manufacturers' instructions. cDNA was generated from 2 μg of RNA using a Superscript II RT-PCR kit (Invitrogen). cDNAs were used as template for standard PCRs with 30 amplification cycles (45 s at 95 °C, 45 s at 65 °C, 1 min at 72 °C). Sequences for forward and reverse primers (569 and 571) to amplify *ORM2* were AACCTGACCATGTGGGAGCAGATT and TTCCCAGCTTAGGAACGACACCAA. Sequences of primers (527 and 528) to amplify *ACT1* were ACGTTCCAGCCTTCTACGTTTCCA and ACGTGAGTAACACCATCACCGGAA. *ORM2* and *ACT1* PCR products were analyzed on ethidium bromide-stained agarose gels and quantitated using ImageJ software.

Electrophoretic Mobility Shift Assay, Western Blot, and Enzyme Assay—To assay Orm phosphorylation, cells were harvested and frozen in liquid nitrogen and trichloroacetic acid, as described (6). Orm1 phosphorylation changes were detected as electrophoretic mobility shifts on 10% polyacrylamide gels using an N-terminal HA-tagged Orm1 construct (pSH14HA). Orm2-TAP mobility shifts were visualized after extended electrophoresis on 12% polyacrylamide gels. For phosphorylation analysis, lysate was prepared by vortexing with glass beads in the presence of trichloroacetic acid, as described previously (6). Alkaline phosphatase digestion of lysates was as described previously (6). 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 mixture and phenylmethylsulfonyl fluoride, as described previously (15). Western blots were visualized with rabbit antibody (to detect the TAP tag) and monoclonal anti-HA (Covance, Princeton, NJ) followed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence detection. Western blots were scanned and quantitated using ImageJ software. 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 β-galactosidase activity was measured as described previously (16). Samples were normalized to protein content assayed by Bradford or BCA (Pierce) assays.

RESULTS

A Calcium-dependent Signaling Pathway Regulates Orm2 Protein Levels—In response to protein misfolding in the ER induced by tunicamycin or DTT, Orm2 protein abundance increased, as shown by Western blot of TAP-tagged Orm2 (Fig. 1*A*) (3, 6). (Orm2-TAP (tagged at the chromosomal locus) is functional because it retains its association with SPT components (3).) The unfolded protein response (UPR), the major transcriptional response to protein misfolding in the ER, requires the sensor Ire1 to activate the transcription factor Hac1 (17). Surprisingly, however, increased Orm2 in response to tunicamycin or DTT was only partially abrogated in *ire1*Δ cells (Fig. 1*A*). To ask whether *ORM2* is a UPR target, a constitutively active Hac1 (spliced Hac1^i) was used to induce UPR in the absence of stressors (18) (Fig. 1*B*). As a control, the ER luminal chaperone Kar2 was induced by Hac1^i or by tunicamycin or DTT addition (Fig. 1*B*, *bottom*). The Orm2 protein level was slightly increased in cells expressing Hac1^i; however, the extent of the increase is far less than that induced by tunicamycin.

Although UPR contributes to increased Orm2 in response to ER stress, another signaling pathway plays an important role. As shown in Fig. 1*A*, Orm2 increase in response to tunicamycin and DTT requires Cch1, a plasma membrane calcium transporter. Increased Orm2 induced by tunicamycin also requires the calcium-dependent signaling molecule, calcineurin; in *cnb1*Δ cells, lacking the regulatory subunit of calcineurin, Orm2 response to tunicamycin is substantially diminished (Fig. 1*A*). Cycloheximide to inhibit protein synthesis prevents tunicamycin-stimulated Orm2 increase, suggesting that decreased Orm2 protein turnover cannot account for the net gain in Orm2 (not shown). Because increased Orm2 also depends on the calcineurin-activated transcription factor, Crz1 (Fig. 1*C*), semiquantitative PCR was used to assess whether *ORM2* is transcriptionally activated by tunicamycin treatment. Fig. 1*D* shows that *ORM2* mRNA transcripts, normalized to *ACT1* transcripts (encoding actin), are increased in cells treated with tunicamycin; by contrast, in *cnb1*Δ cells, *ORM2* transcript increase is abrogated. A summary of quantitated Orm2 protein levels is shown in Fig. 1*E*. In wild-type cells, Orm2 protein level and mRNA transcripts are increased to similar extents by tunicamycin. These results suggest that Orm2 is increased by transcriptional activation, signaled by calcineurin. Our results are consistent with identification of *ORM2* as a target of calcineurin-mediated signaling in a large scale DNA microarray study (19).

As diagrammed in Fig. 1*F*, ER stress leads to activation of the calcium channel Cch1 and calcineurin; in response to stress, many targets of calcineurin participate in membrane remodeling, including lipid components of membranes (20, 21). The calcineurin-mediated response of Orm2 to ER stress was also observed pharmacologically; the calcineurin inhibitor FK506 abolished tunicamycin-induced Orm2 increase (see Fig. 4*B*). The response to ER stress was completely abrogated in *ire1*Δ *cnb1*Δ double mutants (Fig. 1*G*), suggesting that the Orm2 protein level is increased by calcium-dependent signaling augmented by Ire1-dependent signaling.

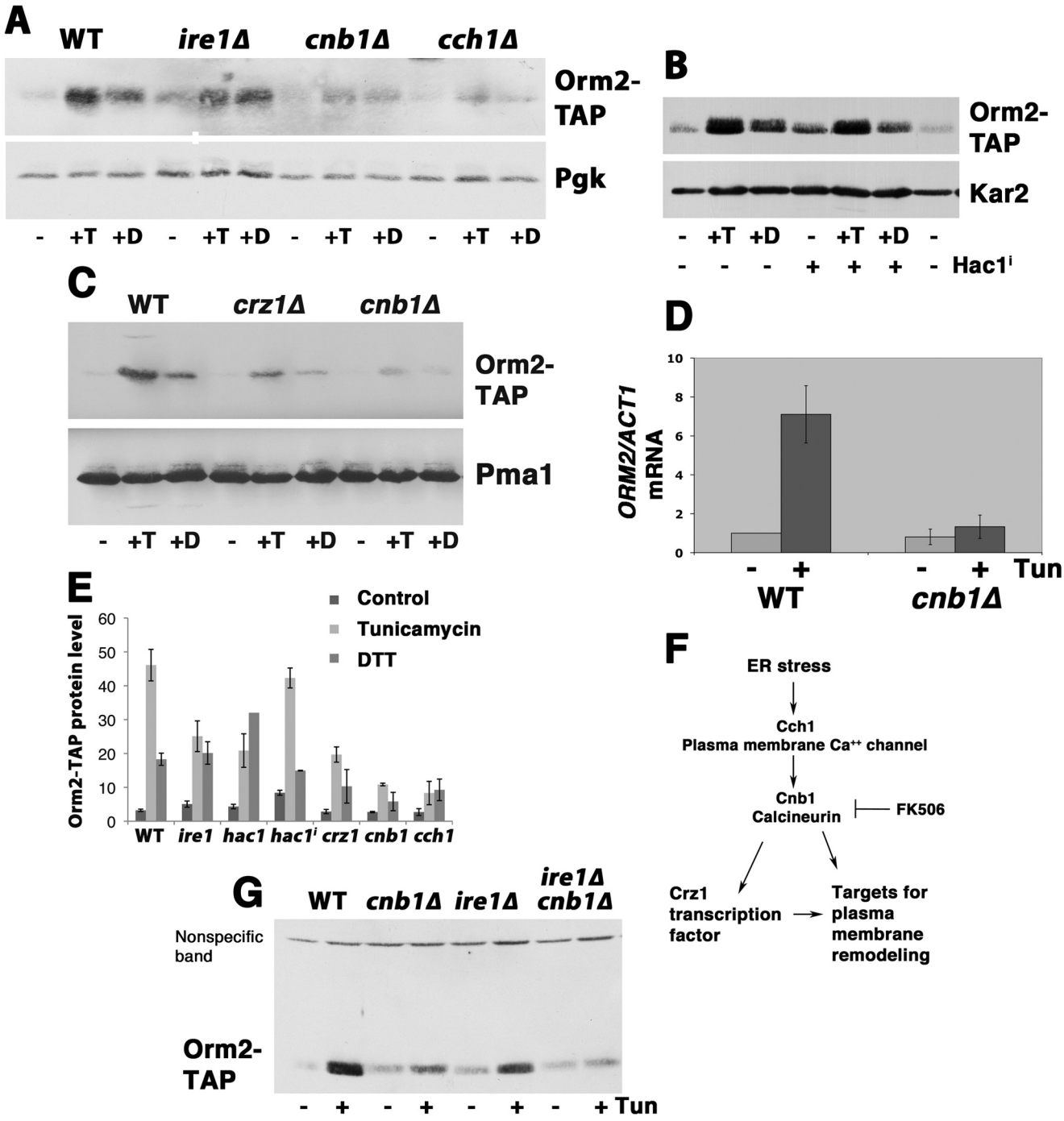

FIGURE 1. **Induction of Orm2 protein by ER stress is dependent on calcium-dependent signaling.** Shown is a Western blot of Orm2-TAP levels in exponentially growing wild-type cells compared with cells treated with tunicamycin (*T*; 1 μg/ml) for 2 h or DTT (*D*; 1 mM) for 2 h. Increased Orm2 protein in response to ER stress is calcium-dependent because it was abrogated in *cch1*Δ (ACX234–1C) and reduced in *cnb1*Δ (ACX204-3B) and *ire1*Δ (ACX195-2D) cells. *A*, Orm2-TAP level (*top*) was increased after treatment with tunicamycin or DTT and partly dependent on Ire1. *Bottom*, phosphoglycerate kinase (*Pgk*) levels are shown as a loading control. *B*, activation of UPR by $Hac1^i$ in the absence of ER stressors had a small effect on Orm2-TAP protein level. Kar2 was blotted as a positive control for UPR activation. *C*, Western blot showing increased Orm2 levels in response to tunicamycin was abrogated in *crz1*Δ (and *cnb1*Δ) cells. Pma1 was blotted as a loading control (*bottom*). *D*, bar graph showing relative *ORM2* mRNA levels in wild-type and *cnb1*Δ cells treated with tunicamycin for 2 h, as measured by semiquantitative PCR and normalized to *ACT1* mRNA. *Error bars*, average of three independent colonies. *E*, summary of quantitated Western blots showing tunicamycin-stimulated increase in Orm2 protein in various deletion strains. *Error bars*, S.E. from $n > 3$ determinations. *F*, schematic diagram showing calcineurin-mediated signaling, activated by ER stress/lipid dysregulation and inhibited by FK506. Calcineurin signaling initiates membrane remodeling by activation of the transcription factor Crz1 and other targets. *G*, Western blot comparing the level of Orm2 induction in *cnb1*Δ and *ire1*Δ and double *cnb1*Δ *ire1*Δ (ACX268-4A) cells.

In addition to activation by protein misfolding, UPR is also induced by cell wall stress (22). To determine whether Orm2 is increased in response to cell wall stress, calcofluor white, a chitin-binding reagent, was added to cells. The Orm2 protein level was slightly increased by calcofluor white (Fig. 2*A*), which strongly activated cell wall stress signaling, as confirmed by induction of *MPK1-lacZ*, a cell wall stress reporter (Fig. 2*B*). By contrast, cell wall stress signaling was not significantly

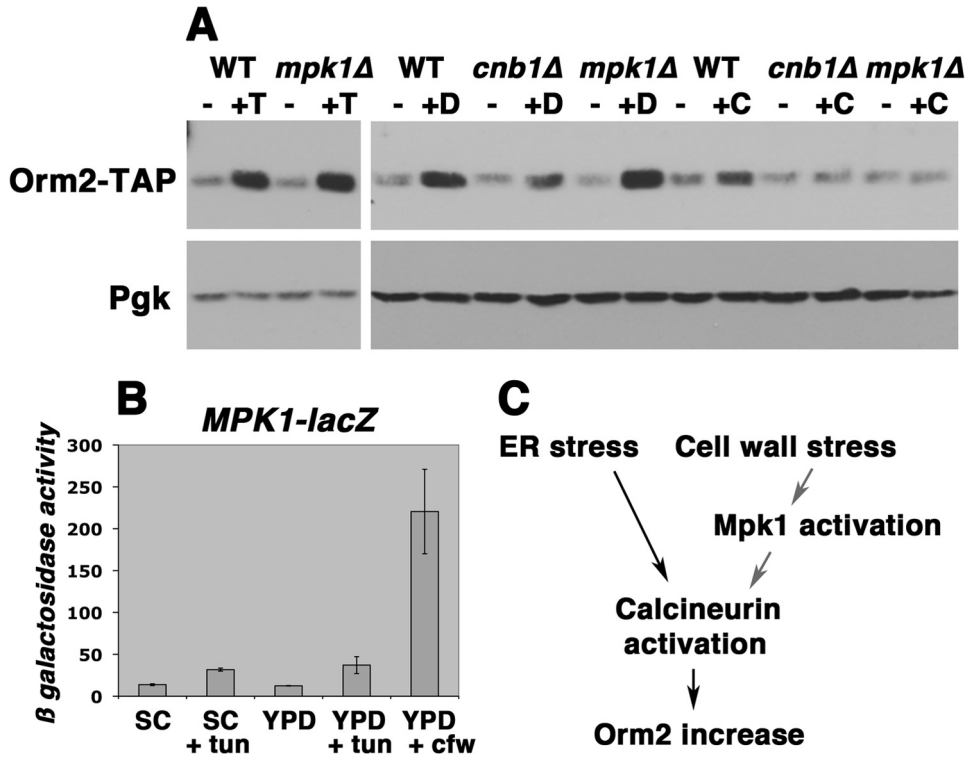

FIGURE 2. **Orm2 protein level is slightly increased by calcofluor white-induced cell wall stress.** Cells were grown overnight to mid-log phase in SC-uracil medium at 30 °C and then shifted to YPD for 6 h before treatment with tunicamycin (1 μg/ml), DTT (1 mM), or calcofluor white (40 μg/ml) for an additional 2 h. Lysates were assayed for both Orm2-TAP levels and activation of cell wall stress signaling. *A*, *top*, Western blot for Orm2-TAP in cells treated with or without tunicamycin (*T*), DTT (*D*), or calcofluor white (*C*). *Bottom*, phosphoglycerate kinase (*Pgk*) blotted as a loading control. *B*, β-galactosidase activity (μmol/min/mg) of an *MPK1-lacZ* reporter to quantitate cell wall stress signaling. Cell wall stress was maximally induced by calcofluor white. *C*, diagram showing that ER stress-induced Orm2 increase is dependent on calcineurin activation, whereas cell wall stress-induced Orm2 increase is signaled through Mpk1 to calcineurin activation.

increased by tunicamycin, and tunicamycin-stimulated Orm2 increase occurred normally even when the cell wall integrity signaling pathway is impaired in *mpk1*Δ cells (23) (Fig. 2*A*). The Orm2 increase elicited by both tunicamycin and calcofluor white was dependent on calcineurin (Fig. 2*A*). As shown in the model in Fig. 2*C*, calcineurin mediates Orm2 increase in response to both ER stress and cell wall stress signaling pathways.

Orm2 Protein Responds to Inositol Levels—Because UPR is induced by inositol deprivation (24) and inositol is a component of the phosphatidylinositol headgroup of complex sphingolipids (25), the Orm2 protein level was examined in response to inositol removal from the medium. As shown in Fig. 3*A*, the Orm2 protein level was increased upon inositol removal. In wild-type cells, the removal of inositol from the medium initiated *de novo* synthesis of inositol by transcription of *INO1*, encoding the rate-limiting enzyme in inositol biosynthesis, as confirmed by *INO1-lacZ* measurement (Fig. 3*B*). In inositol-free medium, derepression of genes encoding phospholipid synthesis enzymes occurs as the repressor Opi1 dissociates from the transcriptional activators Ino2 and Ino4 (26). The Orm2 protein level was not induced in cells in which intracellular inositol did not decrease upon loss of extracellular inositol; when *INO1* is constitutively transcribed (derepressed) in *opi1*Δ cells (Fig. 3*A*) or in cells overexpressing *INO1* (Fig. 3*C*), the Orm2 protein level remained constant in inositol-free medium. By contrast, in *ino2*Δ and *ino4*Δ cells, when derepression of inositol synthesis could not occur, Orm2 was increased constitutively, and when the cells were shifted to inositol-free medium, Orm2 was increased to a greater extent (Fig. 3*A*). Similarly, in *ino1*Δ cells, Orm2 protein was increased in inositol-free medium to a greater extent than that induced in wild-type cells (Fig. 3*C*). It appears that Orm2 protein level is controlled by a signaling pathway that senses decreased intracellular inositol in the absence of extracellular inositol. Orm2 increased ~3-fold in response to inositol-free medium (in comparison with 7–11-fold in response to tunicamycin (Fig. 1*E*)).

Because Orm2 response to tunicamycin is mediated by a calcium- and calcineurin-dependent pathway, we tested whether the same pathway mediates response to inositol-free medium. As shown in Fig. 3*C* (compare *lanes 13* and *14*), Orm2 protein increase in the absence of environmental inositol was dependent on calcineurin because the response was abrogated in *cnb1*Δ cells. Consistently, the response to inositol-free medium was reduced by the calcineurin inhibitor FK506 as well as in the absence of the calcium transporter Cch1 (Fig. 3*D*).

UPR is activated upon loss of inositol from the medium even as *INO1* is derepressed (26, 27) (Fig. 3*E*). Orm2 increase induced in inositol-free medium was not associated with UPR activation; FK506 or loss of Cnb1 or Cch1 inhibited Orm2 response, but calcium- and calcineurin-dependent signaling was not required for increased expression of a *UPRE-lacZ* construct by inositol depletion (Fig. 3*E*).

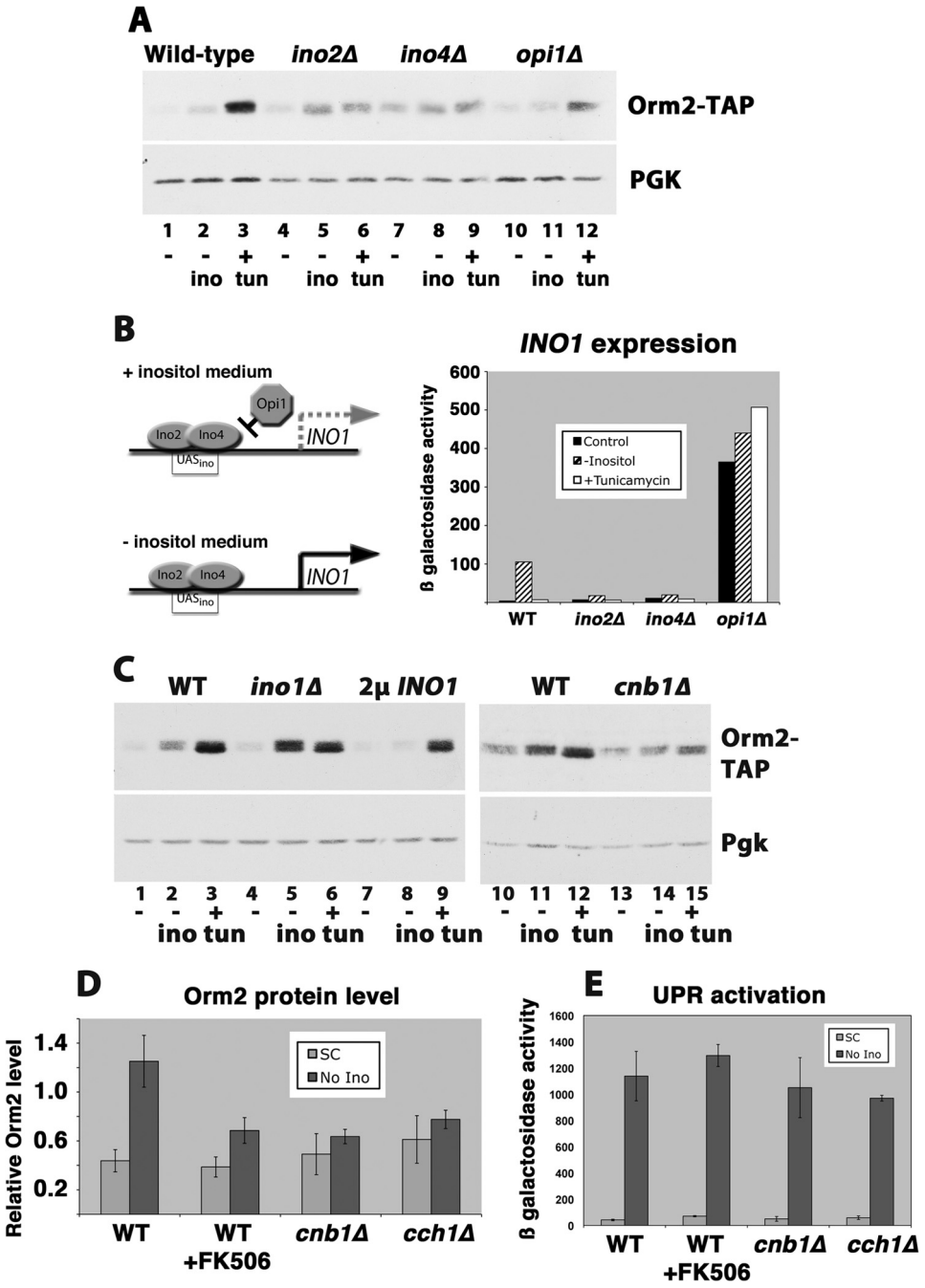

FIGURE 3. **Induction of Orm2 protein in response to inositol removal from the medium.** *A*, Western blot measurement of Orm2-TAP levels in cells incubated in SC medium, inositol-free medium for 3 h, or SC medium plus tunicamycin (*tun*; 1 μg/ml) for 2 h at 30 °C. Increased Orm2 in response to inositol-free medium was prevented by *opi1*Δ; Orm2 was constitutively increased in *ino2*Δ and *ino4*Δ cells. The nitrocellulose filter was reblotted with anti-phosphoglycerate kinase (*PGK*) as a loading control. *B*, *left*, schematic of *INO1* transcriptional regulation. *Right*, derepression of *INO1* in cells incubated in inositol-free medium as measured by an *INO1-lacZ* reporter. β-Galactosidase activity in cell lysate is expressed as μmol/min/mg. *C*, Western blot showing response of wild-type and *cnb1*Δ cells to incubation in inositol-free medium for 3 h. *D*, quantitation of Orm2 response to inositol-free medium of wild-type cells, wild-type cells treated with FK506 (2 μg/ml for 3 h), *cnb1*Δ cells, and *cch1*Δ cells. *Error bars*, S.E. from $n = 3$ determinations. *E*, UPR activation in inositol-free medium. Wild-type, *cnb1*Δ, and *cch1*Δ cells were shifted to inositol-free medium for 3 h, and UPR was assayed by a *UPRE-lacZ* reporter; β-galactosidase activity in cell lysate is expressed as μmol/min/mg.

Orm2 Protein Level Responds to Complex Sphingolipids—To determine whether transcriptional regulation of *ORM2* also acts as a feedback mechanism to control sphingolipid synthesis, mutants defective in different steps of the sphingolipid synthesis pathway were examined. Fig. 4*A* summarizes the key reactions in the sphingolipid synthesis pathway; SPT activity produces the long chain (sphingoid) bases dihydrosphingosine and phytosphingosine (PHS). Subsequent attachment of a very long chain fatty acid to a long chain base produces ceramide. Upon transport of ceramide from ER to Golgi, an inositol headgroup is attached, and the sphingolipid undergoes glycosylation. By contrast with mammalian cells, in which there are hundreds of complex sphingolipids differing in headgroup and/or type and number of sugar groups, there are three complex sphingolipids

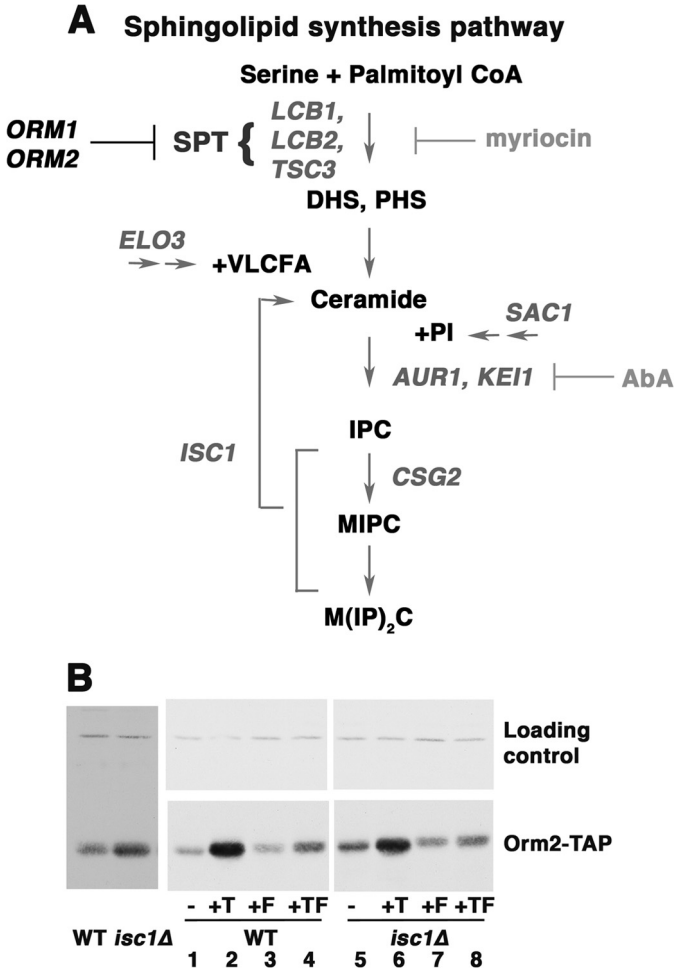

FIGURE 4. **Orm2 protein level is increased in response to accumulation of complex sphingolipids in *isc1*Δ cells.** *A*, diagram of sphingolipid synthesis pathway. *B*, Western blot showing Orm2 protein level in wild-type and *isc1*Δ cells. Cells were grown in SC medium with or without tunicamycin (*T*; 1 μg/ml for 2 h), FK506 (*F*; 2 μg/ml for 2 h), or tunicamycin plus FK506 (*TF*). Orm2 protein level was constitutively increased in *isc1*Δ cells in comparison with wild-type cells (*left* and *right*, compare *lanes 1* and *5*). The Orm2 protein level in *isc1*Δ cells was decreased by 2 h after FK506 addition (*right*, compare *lanes 5* and *7*).

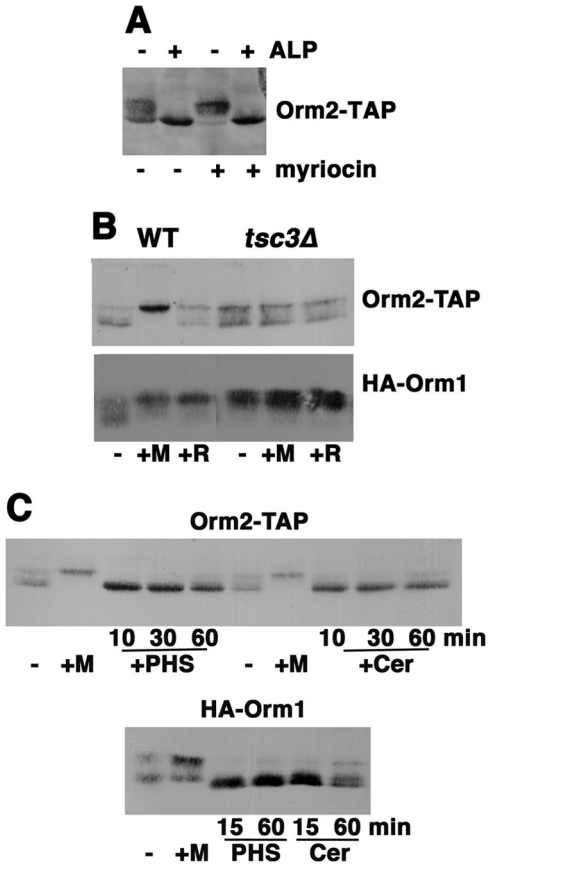

FIGURE 5. **Phosphorylation of the Orm proteins responds to early intermediates in the sphingolipid synthesis pathway.** *A*, Western blot of Orm2-TAP; electrophoretic mobility reflects Orm2 phosphorylation. Cells were treated with or without myriocin (0.15 μg/ml) for 1 h. Lysate was prepared in the presence of TCA and treated with or without alkaline phosphatase (*ALP*) for 1 h at 37 °C, as described previously (6). The fastest migrating band appears unphosphorylated because its mobility remained the same after alkaline phosphatase treatment. *B*, Western blot showing electrophoretic mobilities of HA-Orm1 and Orm2-TAP from cells with or without 0.15 μg/ml myriocin (*M*) or 20 μM rapamycin (*R*) for 1 h at 30 °C. In *tsc3*Δ cells, phosphorylation of both Orm proteins was constitutively increased. *C*, HA-Orm1 and Orm2-TAP proteins were dephosphorylated in response to the addition of myriocin (1 h) or PHS (20 μM) or C_2-ceramide (*Cer*; 10 μM) for various times.

in yeast, each with an inositol headgroup: inositol phosphorylceramide (IPC), mannose inositol phosphorylceramide, and mannose diinositol phosphorylceramide (25).

In *isc1*Δ cells, defective in sphingolipid phospholipase C, complex sphingolipids are accumulated (28) (Fig. 4*A*). Coincident with accumulated complex sphingolipids in *isc1*Δ cells, there was a slight increase in Orm2 protein level (Fig. 4*B*). Calcineurin mediates Orm2 increase signaled by accumulated sphingolipids because the addition of FK506 reduced increased Orm2 in *isc1*Δ cells (Fig. 4*B*, compare *lanes 1* and *7*). As expected, FK506 also reduced Orm2 response to tunicamycin (Fig. 4*B*, compare *lanes 2* and *4*). Because sphingolipid synthesis is repressed when Orm2 protein is increased (6), it appears that increased Orm2 abundance is a feedback mechanism to down-regulate sphingolipid synthesis in *isc1*Δ cells. No other significant change in Orm2 protein level was observed in other sphingolipid synthesis mutants (not shown).

Orm Phosphorylation Responds to Flux through the Sphingolipid Synthesis Pathway—In addition to changes in Orm2 protein level, sphingolipid synthesis is regulated by phosphorylation of both Orm proteins in response to environmental conditions (6–8). Orm phosphorylation has also been suggested to respond to downstream sphingolipid biosynthetic intermediates (4). To identify the sphingolipid intermediate(s) that signal Orm response, combined genetic and chemical approaches were taken to study Orm phosphorylation when the sphingolipid synthesis pathway is perturbed. The extent of phosphorylation of HA-tagged Orm1 was assayed by changes in electrophoretic mobility, as described previously (6). Similarly, electrophoretic mobility changes of Orm2-TAP (tagged at the chromosomal locus) reflect changes in Orm2 phosphorylation. Reduced mobility of Orm2-TAP and HA-Orm1 reflects hyperphosphorylation because alkaline phosphatase treatment collapsed slower migrating bands to a single fast migrating band (Figs. 5*A* and 7*D*).

In cells deleted of *TSC3*, encoding the small stimulatory subunit of SPT, there is a severe reduction in SPT activity (29). In

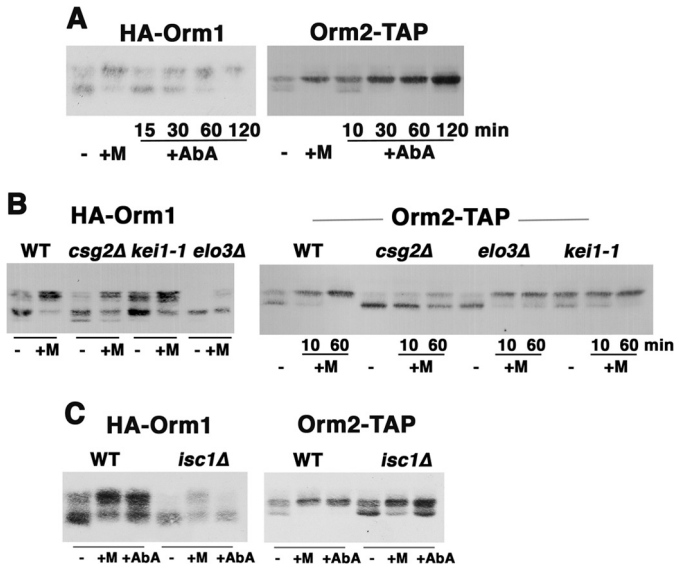

FIGURE 6. **Phosphorylation of the Orm proteins in response to sphingolipid metabolites in the distal sphingolipid synthesis pathway.** *A*, Western blot showing electrophoretic mobility changes of HA-Orm1 and Orm2-TAP in exponentially growing wild-type control cells in comparison with cells incubated with myriocin (0.15 μg/ml) for 1 h or for the indicated times with AbA (2 μg/ml). *B*, electrophoretic mobility assay of Orm proteins in wild-type, *elo3*Δ, *csg2*Δ, and *kei1-1* cells with or without myriocin for 10 min and/or 1 h. *C*, electrophoretic mobility assay of Orm proteins in wild-type and *isc1*Δ cells. Cells were treated with or without myriocin or AbA for 1 h.

*tsc3*Δ cells, mobility shifts indicate increased phosphorylation of both HA-Orm1 and Orm2-TAP (Fig. 5*B*). Constitutive phosphorylation of Orm1 appeared maximal in *tsc3*Δ cells because its mobility was similar to that stimulated by the addition of myriocin for 1 h. These results support a model in which Orm1 phosphorylation is a compensatory response to SPT activity acutely inhibited by myriocin or chronically depressed in *tsc3*Δ cells. Phosphorylation of Orm2 was also constitutively increased in *tsc3*Δ cells; however, Orm2 phosphorylation was not further increased to the maximal extent by myriocin. One possible explanation for an apparent diminished response to myriocin is that Orm2 phosphorylation may respond to different extents to multiple downstream sphingolipid intermediates (discussed further below).

A time course of Orm phosphorylation upon the acute addition of downstream SPT products, long chain base (PHS) and ceramide (a C-2 ceramide analog), is shown in Fig. 5*C*. Both Orm1 and Orm2 underwent rapid hypophosphorylation, suggesting a direct effect of the downstream intermediates on the Orm-SPT complex. To examine whether Orm phosphorylation is also adjusted in response to the level of complex sphingolipids, aureobasidin A (AbA) was added to inhibit attachment of the inositol headgroup to ceramide to form IPC (Fig. 4*A*) (30). Strikingly, AbA induced increased phosphorylation of both Orm1 and Orm2 (Fig. 6*A*); these results suggest that decreased IPC levels also feed back to the Orm proteins to increase SPT activity. Feedback control of SPT by IPC was studied further in cells bearing a temperature-sensitive allele of *KEI1*, encoding a subunit of IPC synthase (11). The *kei1-1* mutant was analyzed at the semipermissive temperature (30 °C) to avoid effects on sphingolipid synthesis that occur at 37 °C (31). As shown in Fig. 6*B*, phosphorylation of Orm1 and Orm2 was constitutively increased in *kei1-1* cells, supporting the idea that both Orm proteins respond to IPC levels.

In the sphingolipid synthesis pathway, Elo3 catalyzes the last of a series of reactions to synthesize very long chain fatty acids required for the formation of ceramide, causing a backlog in the pathway at ceramide synthesis (Fig. 4*A*) (25, 32). In *elo3*Δ cells, high levels of PHS are accumulated (32). Fig. 6*B* shows that in the absence of Elo3, the Orm proteins were dephosphorylated. The response to myriocin was impaired (Fig. 6*B*) because constitutively elevated PHS in *elo3*Δ cells may interfere with the ability of myriocin to signal increased Orm phosphorylation. A similar phenotype was seen in *csg2*Δ cells lacking a calcium-binding protein required in catalyzing mannosylation of IPC; IPC is accumulated in *csg2*Δ cells (25, 33). In *csg2*Δ cells, both Orm proteins were constitutively dephosphorylated in comparison with wild-type cells (Fig. 6*B*). The response to myriocin by hyperphosphorylation was impaired in *csg2*Δ cells so that neither Orm protein can achieve maximal phosphorylation (Fig. 6*B*). It seems possible that the effect of myriocin to decrease downstream products is quelled by elevated levels of IPC in *csg2*Δ cells (34).

Further evidence for Orm response to IPC is shown in Fig. 6*C*. Without complex sphingolipase activity in *isc1*Δ cells (28), Orm1 was constitutively dephosphorylated, and to a lesser extent, Orm2 was also constitutively dephosphorylated. These responses to accumulated complex sphingolipids are consistent with feedback repression of sphingolipid synthesis (Fig. 6*C*). In *isc1*Δ cells, hyperphosphorylation in response to AbA or myriocin addition for 1 h was impaired to a greater and lesser extent for Orm1 and Orm2, respectively (Fig. 6*C*); the effect of the inhibitors to decrease IPC production was probably blunted by built-up complex sphingolipids. Together, these data suggest that feedback regulation of SPT activity is mediated via Orm phosphorylation by multiple downstream products in the sphingolipid synthesis pathway, including long chain base, ceramide, and the complex sphingolipid IPC.

TOR Signaling Regulates Orm Phosphorylation—Phosphorylation of Orm1 and Orm2 is regulated by the TOR signaling pathway via Npr1 and Ypk1, respectively, representing TORC1 and TORC2 branches (6, 8, 35). Hyperphosphorylation of Orm1 in response to inhibition of sphingolipid synthesis by myriocin or AbA treatment was abrogated in *npr1*Δ cells (Fig. 7*A*, *left*). Orm2 hyperphosphorylation in response to myriocin or AbA was diminished in *ypk1*Δ cells (Fig. 7*A*, *right*). Thus, feedback response of Orm phosphorylation to flux through the sphingolipid synthesis pathway occurs via TOR pathway kinases.

In addition to feedback regulation, Orm1 phosphorylation is also regulated by Npr1 in response to nutritional status (6, 9). Orm1 phosphorylation is increased upon rapamycin treatment to inhibit TORC1, mimicking amino acid starvation (6, 9). Although Orm1 phosphorylation responded maximally to rapamycin, Orm2 response was reduced (Fig. 7*B*, *left* and *right*, compare *lanes 3*). Orm2 response to rapamycin was minimal in comparison with its feedback response (as assayed by its response to myriocin treatment) (Fig. 7*B*, *right*, compare *lanes 2* and *3*). Indeed, Orm2 response to rapamycin was further diminished in *orm1*Δ cells by dephosphorylation

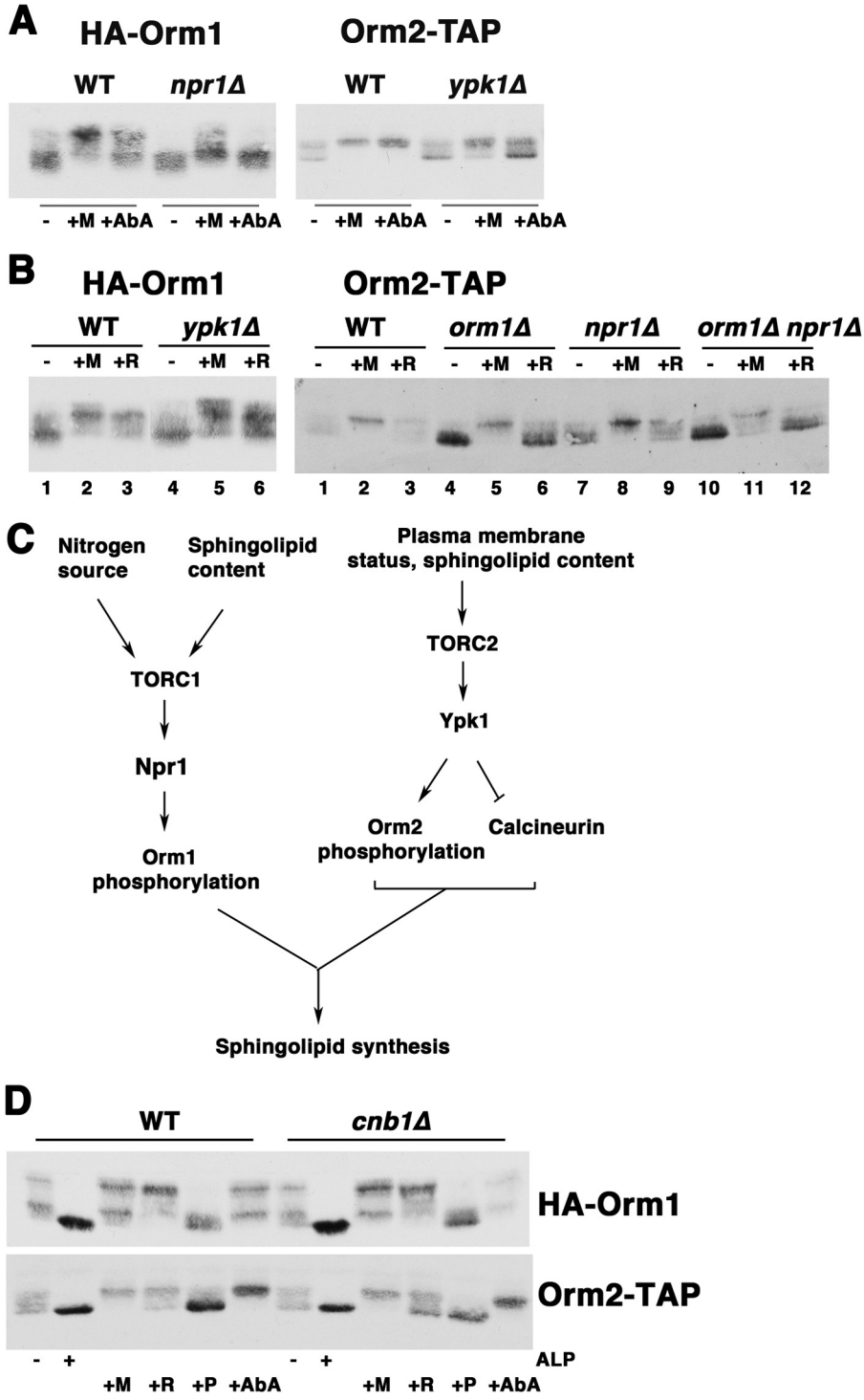

FIGURE 7. **Regulation of Orm protein phosphorylation of the Orm protein by TORC1 and TORC2 signaling pathways.** *A*, electrophoretic mobility assay of HA-Orm1 and Orm2-TAP phosphorylation. Cells were treated with or without myriocin (0.15 μg/ml) or AbA (2 μg/ml) for 1 h. *Left*, *orm1*Δ (HXX1-7B) and *orm1*Δ *npr1*Δ (ACX251-10B) bearing pSH14HA. *Right*, wild-type (HXX1-7C) and *ypk1*Δ (ACX233-1C) cells with chromosomal *ORM2* tagged with a TAP tag. *B*, Western blot of HA-Orm1 and Orm2-TAP phosphorylation in response to rapamycin treatment. Cells were treated with myriocin (+*M*; 0.15 ng/ml) or rapamycin (+*R*; 20 μM) for 1 h. *Left*, wild-type (HXX1-7C) and *ypk1*Δ cells bearing pSH14HA. *Right*, strains with chromosomal *ORM2* tagged with TAP: wild type (ACX251-9B), *orm1*Δ (ACX190-3A), *npr1*Δ (251-6D), and *orm1*Δ *npr1*Δ (ACX251-9A). *C*, schematic diagram showing signaling pathways involved in regulating sphingolipid synthesis. TORC1 plays a major role in regulating Orm1 phosphorylation via Npr1. Regulation of Orm2 activity by phosphorylation is primarily under the control of TORC2 via Ypk1. Calcineurin signaling regulates Orm2 protein levels; increased Orm2 represses SPT activity (6). *D*, Western blot of Orm phosphorylation in wild-type and *cnb1*Δ cells. Cells were incubated with 0.15 μg/ml myriocin (+*M*), 20 μM rapamycin (+*R*), 20 μM phytosphingosine (+*P*), or 2 μg/ml aureobasidin A (+*AbA*) for 1 h at 30 °C. Lysates were treated with or without alkaline phosphatase (*ALP*) for 1 h at 37 °C as indicated.

(Fig. 7*B*, *right*, compare *lane 6* with *lanes 3* and *9*), suggesting the possibility that Orm2 response to rapamycin occurs indirectly via Orm1.

Fig. 7*C* presents a model in which Orm1 is a major focus of TORC1 regulation via Npr1. Orm2 phosphorylation remained unaffected in *npr1*Δ cells (Fig. 7, compare *A* (*left*) with *B* (*right*)).

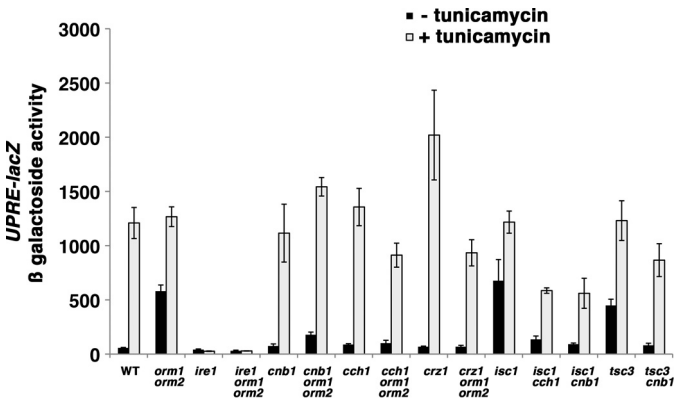

FIGURE 8. **ER stress in *orm1*Δ *orm2*Δ cells is signaled via calcium and calcineurin.** Constitutive UPR activation in *orm1*Δ *orm2*Δ cells is dependent on calcium- and calcineurin-dependent signaling. Exponentially growing cells bearing a *UPRE-lacZ* reporter (pJC104) (52) were grown in the presence or absence of tunicamycin (1 μg/ml) for 2 h. β-Galactosidase activity was measured in cell lysate, as described (6). *Error bars*, S.E.

Orm2 is a major focus of TORC2 via Ypk1, and Orm1 phosphorylation was minimally affected in *ypk1*Δ cells (Fig. 7, compare *A* (*right*) with *B* (*left*)).

Because TOR and calcineurin have a mutually antagonistic relationship (36), we tested whether Orm phosphorylation is regulated by calcineurin's phosphatase activity. As shown in Fig. 7*D*, phosphorylation of HA-Orm1 and Orm2-TAP were examined in *cnb1*Δ cells. Phosphorylation of both Orm proteins appeared identical in wild-type and *cnb1*Δ cells; both Orm proteins displayed increased phosphorylation in response to myriocin and AbA addition to inhibit SPT and IPC synthase, respectively. In wild-type and *cnb1*Δ cells, both Orm proteins were dephosphorylated upon the addition of PHS (Fig. 7*D*). Thus, calcineurin does not play a significant role in regulating Orm phosphorylation, although it does modulate Orm2 protein level (Fig. 1).

UPR Activation in Response to Lipid Dysregulation Is Dependent on Calcium and Calcineurin—In the absence of regulation via the Orm proteins, perturbation of lipid homeostasis causes ER stress, and *orm1*Δ *orm2*Δ cells have constitutive activation of UPR and impaired growth in the presence of tunicamycin (3) (Fig. 8). Constitutive UPR was prevented in *ire1*Δ *orm1*Δ *orm2*Δ cells because Ire1 is required for UPR activation, as measured by a *UPRE-lacZ* reporter (Fig. 8) (3). Because calcineurin regulates Orm2 abundance and lipid synthesis (20), a role for calcineurin was tested in UPR activation in *orm1*Δ *orm2*Δ cells. Fig. 8 shows that constitutive UPR requires both calcium and calcineurin because its induction was prevented in *cch1*Δ *orm1*Δ*orm2*Δ and *cnb1*Δ *orm1*Δ *orm2*Δ cells. Moreover, calcium- and calcineurin-mediated signaling to activate the Crz1 transcription factor is required for constitutive UPR because it was abolished in *crz1 orm1*Δ *orm2*Δ cells (Fig. 8). UPR was also constitutively activated when complex sphingolipids were accumulated in *isc1*Δ cells as well as when sphingolipid synthesis was constitutively decreased in *tsc3*Δ cells (Fig. 8). Strikingly, calcium and calcineurin were required to mediate UPR signaling in *isc1*Δ and *tsc3*Δ cells as well (Fig. 8), consistent with a role for this signaling pathway to maintain sphingolipid homeostasis. UPR induction by tunicamycin treatment (which increases both protein misfolding and calcineurin signaling) was not significantly impaired in any of the tested mutants except for *ire1*Δ cells (Fig. 8). Thus, calcium- and calcineurin-mediated signaling appear to elicit the UPR specifically in response to sphingolipid dysregulation.

DISCUSSION

Regulation of sphingolipid synthesis is mediated by phosphorylation of Orm1 and Orm2 (6, 8) as well as by up-regulation of Orm2 protein levels (6) (Fig. 1). A major finding of this paper is that ER stress signaling to increase Orm2 abundance occurs via a calcium- and calcineurin-dependent pathway. Although UPR activation is the major response to ER stress, activated $Hac1^{i}$ in the absence of tunicamycin or DTT stressor induces only a small increase in Orm2; UPR makes only a partial contribution to inducing Orm2. Previous work has shown that ER stress triggers calcium influx through the Cch1-Mid1 calcium channel and calcineurin activation; this calcium-calcineurin signaling pathway acts coordinately with the UPR and ensures cell survival during ER stress (21, 37–39). In addition to increasing Orm2 to repress SPT, calcium/calcineurin-mediated signaling regulates late steps in sphingolipid synthesis: the mannosylation of IPC to mannose inositol phosphorylceramide via Csg1/Sur1 and Csg2 (19) and complex sphingolipid breakdown via Isc1 activity (40). Together, sphingolipids and calcineurin regulate Slm1 and Slm2 proteins, which are proposed to act as integrators of different inputs in the TORC2 protein kinase network (40–42).

Another environmental stimulus that induces increased Orm2 is loss of inositol from the medium (Fig. 3). Induction of Orm2 protein by inositol-free medium is independent of *INO1* derepression and inositol biosynthesis because Orm2 is increased in *ino1*Δ, *ino2*Δ, and *ino4*Δ cells. Instead, increased Orm2 in response to loss of medium inositol is triggered by calcineurin-mediated signaling (Fig. 3*C*); our results are in agreement with a previous report showing activation of calcineurin signaling in response to inositol starvation (43). Several genes have been identified that are under negative control by inositol (44) and the transcription factors Ino2 and Ino4 (45); like these, *ORM2* appears transcriptionally activated in *ino2*Δ or *ino4*Δ cells (Fig. 3*A*). Signaling via calcineurin affects plasma membrane remodeling in response to a variety of stress conditions (20). Control of sphingolipid synthesis upon depletion of intracellular inositol and perturbation of phospholipid homeostasis underscores the link to the lipid component of calcineurin-mediated membrane restructuring.

The Orm proteins participate in a feedback mechanism to modulate SPT activity in response to flux through the sphingolipid synthesis pathway (4). We now show that multiple intermediates in the sphingolipid synthesis pathway serve as signals to regulate Orm phosphorylation, including long chain base, ceramide, and complex sphingolipid. Our results show that both Orm proteins become hypophosphorylated upon the addition of the long chain base PHS (in agreement with a previous report (8)) as well as a C_2-ceramide (Fig. 5). Hyperphosphorylation of the Orm proteins is triggered by the addition of myriocin to produce an acute drop in long chain base and ceramide levels. Complex sphingolipid is also a feedback signal to

Orm phosphorylation because the addition of AbA to decrease IPC acutely elicits hyperphosphorylation of both Orm proteins (Fig. 6). Conversely, accumulation of IPC and other complex sphingolipids in *isc1*Δ cells generates constitutively dephosphorylated Orm phosphorylation (Fig. 6).

In several mutants impaired in different steps in the sphingolipid synthesis pathway, there is a blunted response of Orm phosphorylation to myriocin and AbA. These results are consistent with contradictory signals; for instance, in *csg2*Δ cells, constitutively high IPC levels conflict with an AbA-mediated IPC decrease, resulting in impaired response to AbA (Fig. 6). Strikingly, in addition to regulating phosphorylation, IPC accumulation modulates Orm2 protein level (Fig. 4). Thus, build-up of the end product down-regulates the first committed step of the pathway via two different regulatory mechanisms to decrease Orm phosphorylation and increase Orm2 protein.

Feedback regulation of Orm phosphorylation is mediated by both branches of the TOR signaling pathway (Fig. 7). A recent paper reported that distinct sites on Orm1 are phosphorylated in response to sphingolipid status (after myriocin or AbA treatment) or nutritional status (after rapamycin treatment) (9). Previously, we showed that the Npr1 protein kinase, downstream of TORC1, regulates Orm1 phosphorylation (6). Abrogation of HA-Orm1 phosphorylation in *npr1*Δ cells supports the idea that Npr1 mediates, in large part, response to both nutritional status (6) and feedback response (Fig. 7*A*) (6). By contrast with its effect on Orm1, Npr1 appears to play a less significant role in Orm2 regulation; in *npr1*Δ cells, feedback phosphorylation of Orm2-TAP is essentially unaffected (Fig. 7, *A* and *B*). Orm2 appears largely regulated by TORC2. There is a slight Orm2 response to the TORC1 inhibitor rapamycin (Fig. 7*B*), but feedback phosphorylation of Orm2-TAP is substantially diminished in the absence of the AGC family kinase, Ypk1, acting in the TORC2 pathway (Fig. 7*A*) (7, 8). Conversely, feedback Orm1 phosphorylation and Orm1 response to rapamycin is not detectably affected in *ypk1*Δ cells (Fig. 7*B*). Thus, Orm1 and Orm2 are overlapping but distinct targets of TORC1 and TORC2, respectively. A model is shown in Fig. 7*C* depicting Orm regulation via branches of the TOR signaling network.

In the absence of the Orm proteins, dysregulation of SPT and sphingolipid homeostasis results in pleiotropic phenotypes (3), including constitutive activation of the UPR (Fig. 8) (3). UPR activation in *orm*Δ cells requires calcium influx via Cch1 and calcineurin. By contrast, UPR activation by tunicamycin is calcineurin-independent, suggesting that calcineurin-mediated signaling in *orm1*Δ *orm2*Δ cells is not activated by protein misfolding *per se*. Instead, UPR induction through calcium and calcineurin signaling appears to reflect a reaction to lipid perturbation, and also requires Ire1. Our result is consistent with distinct domains of Ire1 responding to different ER stresses (46). More importantly, our results support accruing evidence that ER homeostasis is maintained by cross-talk between UPR and other signaling pathways (47, 48).

Calcium and calcineurin are at the hub of a signaling network that maintains sphingolipid homeostasis. TORC2 has been reported to regulate ceramide synthesis in a calcineurin-dependent manner (49). Our observations here suggest that TORC2, as a negative regulator of calcineurin (50, 51), probably regulates sphingolipid synthesis by calcineurin-mediated modulation of Orm2 levels (Fig. 1). Strikingly, calcineurin does not appear to regulate Orm phosphorylation (Fig. 7*D*). Calcineurin-mediated regulation of sphingolipid homeostasis contributes to our understanding of how the plasma membrane landscape is sculpted in response to cellular needs and environmental signals. The importance of sphingolipids in membrane biogenesis and cell growth is underscored by regulation by both branches of TOR signaling.

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