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# Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control

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**Yeast members of the ORMDL family of endoplasmic reticulum (ER) membrane proteins play a central role in lipid homeostasis and protein quality control. In the absence of yeast Orm1 and Orm2, accumulation of long chain base, a sphingolipid precursor, suggests dysregulation of sphingolipid synthesis. Physical interaction between Orm1 and Orm2 and serine palmitoyltransferase, responsible for the first committed step in sphingolipid synthesis, further supports a role for the Orm proteins in regulating sphingolipid synthesis. Phospholipid homeostasis is also affected in *orm1*Δ *orm2*Δ cells: the cells are inositol auxotrophs with impaired transcriptional regulation of genes encoding phospholipid biosynthesis enzymes. Strikingly, impaired growth of *orm1*Δ *orm2*Δ cells is associated with constitutive unfolded protein response, sensitivity to stress, and slow ER-to-Golgi transport. Inhibition of sphingolipid synthesis suppresses *orm1*Δ *orm2*Δ phenotypes, including ER stress, suggesting that disrupted sphingolipid homeostasis accounts for pleiotropic phenotypes. Thus, the yeast Orm proteins control membrane biogenesis by coordinating lipid homeostasis with protein quality control.**

membrane biogenesis | sphingolipid synthesis | unfolded protein response



Protein quality control at the endoplasmic reticulum (ER) involves a number of mechanisms to promote proper folding and assembly of newly synthesized proteins (1). The unfolded protein response (UPR) ameliorates ER stress that results from accumulation of misfolded proteins or perturbation in lipid homeostasis. Upon induction of UPR, there is transcriptional activation of target genes encoding factors that assist protein folding, remove misfolded proteins, and promote lipid synthesis (2).

The ER serves as the main site for synthesis of three major classes of membrane lipids: sphingolipids, phospholipids, and sterols. Sphingolipid synthesis is initiated at the ER by serine palmitoyltransferase (SPT), which catalyzes the condensation of serine and palmitoyl CoA to produce long chain bases (3). Biosynthesis of phospholipids and sterols occurs largely at the ER, and homeostasis of these lipids is regulated by ER-localized sensors via transcriptional and posttranscriptional mechanisms (4). To maintain balance in membrane lipid constituents, cross-talk between sterol and sphingolipid pathways has been extensively documented; sphingolipid and phospholipid pathways are linked through common biosynthetic metabolites and also signaling via these metabolites.

Membrane biogenesis involves integration of protein quality control with lipid homeostasis to ensure a proper balance of proteins and lipids. The mechanistic ties between protein quality control and maintenance of sphingolipid, phospholipid, and sterol homeostasis are poorly understood. In this study, we describe two ER membrane proteins that regulate membrane biogenesis. Yeast Orm1 and Orm2 belong to a unique ORMDL family (5). The physiologic importance of the Orm proteins is underscored by recent reports that the human gene *ORMDL3* is an asthma susceptibility gene (6). In yeast, a role for the Orm proteins in regulating sphingolipid synthesis is indicated by long chain base accumulation in *orm1*Δ *orm2*Δ cells, and physical interaction between the Orm proteins and SPT, responsible for the first committed step in sphingolipid synthesis. ER quality control is impaired in the absence of Orm1 and Orm2: UPR is constitutively activated, stress resistance is defective, and ER-to-Golgi transport is slowed. Because ER stress phenotypes in *orm1*Δ *orm2*Δ cells are suppressed by inhibiting sphingolipid synthesis, we suggest that Orm1 and Orm2 coordinate lipid homeostasis with protein quality control in the ER.

## Results

**ER Stress Response.** Orm1 and Orm2 are localized to the ER (7), and on the basis of predicted amino acid sequence, have 222 and 216 residues, respectively, share ≈70% identity with each other, and have similar topology with four transmembrane domains. To confirm a previous report that deletion of *ORM1* and *ORM2* results in susceptibility to agents that increase protein misfolding in the ER (5), we tested cell growth in various media. At 30 °C, *orm1*Δ *orm2*Δ cells have a growth defect (Fig. 1*A*). In the presence of tunicamycin, an inhibitor of N-linked glycosylation, *orm1*Δ *orm2*Δ cells display further impaired growth. *ORM1* and *ORM2* have overlapping function, because overexpression of *ORM1* can suppress the tunicamycin sensitivity of *orm1*Δ *orm2*Δ cells. The sensitivity of *orm1*Δ *orm2*Δ cells to protein misfolding was examined further by expressing the misfolded luminal protein CPY* using a *pGAL-CPY** construct. Fig. 1*A* shows defective growth of *orm1*Δ *orm2*Δ cells on galactose when CPY* is overexpressed (Fig. 1*A*), but not on glucose.

ER stress in *orm1*Δ *orm2*Δ cells was assessed by measuring UPR using a *UPRE-lacZ* reporter. In wild-type cells, β-galactosidase activity is constitutively low but is increased after addition of the reducing agent DTT (Fig. 1*B*). UPR is constitutively elevated in *orm1*Δ *orm2*Δ cells, indicating increased protein misfolding in the ER. DTT addition to increase protein misfolding in the ER produces a robust UPR response in *orm1*Δ *orm2*Δ cells (Fig. 1*B*).

Additional stress responses were assayed in *orm1*Δ *orm2*Δ cells. Reporter constructs were used to assay cell wall stress (*MPK1-lacZ*) (8) and heat shock response (HSR) (*HSE-lacZ*) (9). Cell wall perturbation initiates transcriptional changes to improve cell integrity; HSR mediated by the transcription factor Hsf1 results in up-regulation of chaperones and the ubiquitin/proteasome pathway. Constitutive activation of these stress responses was not detected in *orm1*Δ *orm2*Δ cells. However, *orm1*Δ *orm2*Δ mutants seem impaired in the extent of cell wall stress response to calcofluor white as well as HSR induced by incubation at 37 °C (Fig. S1).

Author contributions: S.H., R.S., and A.C. designed research; S.H., M.A.L., and A.C. performed research; S.H., M.A.L., R.S., and A.C. analyzed data; and R.S. and A.C. wrote the paper.

The authors declare no conflict of interest.

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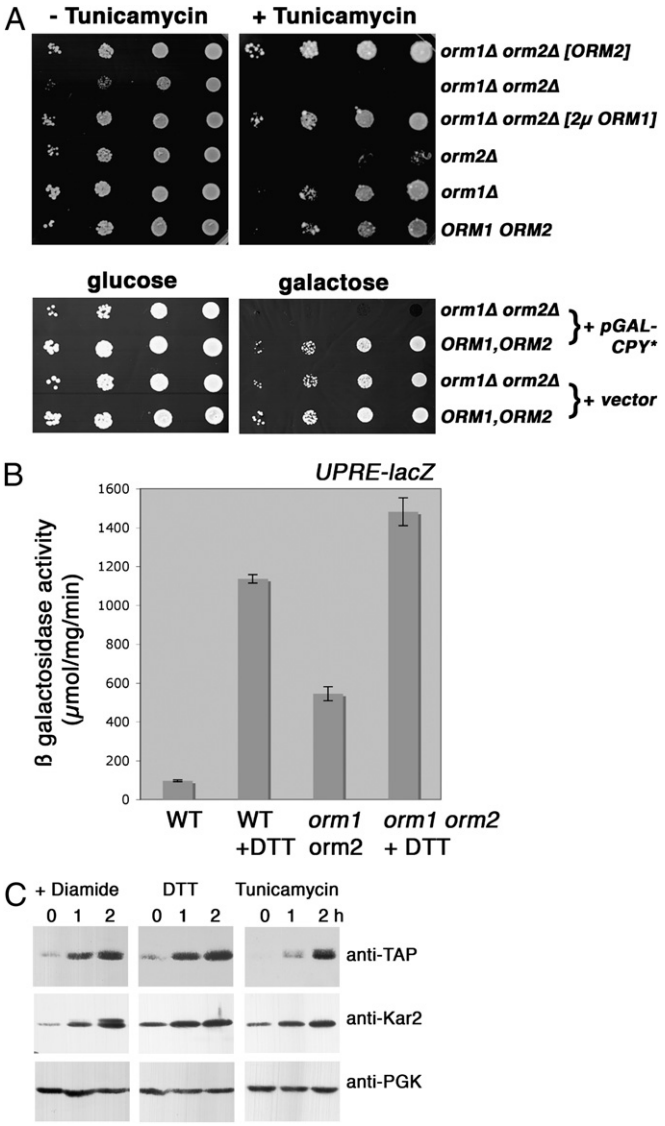


**Fig. 1.** Stress response upon loss of Orm1 and Orm2. (*A*) Loss of Orm1 and Orm2 causes sensitivity to stress. Cells were normalized to 0.1 $OD_{600}$/mL and spotted onto plates containing 1 μg/mL tunicamycin in SC medium with 2% glucose, and incubated for 2 to 3 days at 30 °C. Tunicamycin sensitivity of *orm1*Δ *orm2*Δ cells is complemented by *ORM2* expressed from a centromeric plasmid (pSH15) and suppressed by high-copy *ORM1* (pSH16). For overexpression of CPY*, cells bearing vector or *pGAL-CPY** (pES67) were spotted onto plates with 2% glucose or 2% galactose. (*B*) UPR is induced constitutively in *orm1*Δ *orm2*Δ cells. Wild-type (HXX1-7C) and *orm1*Δ*orm2*Δ (HXX1-7D) cells bearing a *UPRE-lacZ* reporter (pJC104) were incubated with or without 5 mM DTT for 1 h at 30 °C. Cell lysate was prepared and β-galactosidase assays were performed as previously described (45). Assays were performed in duplicate on at least three independent colonies. (*C*) Orm2 is increased in response to stress. Exponentially growing cells were incubated with or without tunicamycin (1 μg/mL), DTT (2 mM), or diamide (2 mM) for 1 h and 2 h at 30 °C in SC medium. Samples were normalized to lysate protein, and TAP-tagged Orm2 was analyzed by Western blot.

Because UPR is constitutively active in *orm1*Δ *orm2*Δ cells, and *ORM2* was previously identified as a UPR target in a genome-wide microarray assay (2), we tested the possibility that Orm2 is involved in protection from stress conditions. Cells were analyzed with a functional Tandem Affinity Purification (TAP)-tagged Orm2 expressed from its chromosomal locus. Orm2-TAP protein was quantitated after cells were treated for 1 to 2 h with DTT or tunicamycin to increase protein misfolding, or diamide to produce oxidative stress (Fig. 1*C*). Orm2 protein is significantly increased, indicating that it is up-regulated by UPR. These results are consistent with results from global microarray studies (2, 10). The ER chaperone Kar2, a known UPR target (2), is also increased by DTT, tunicamycin, and diamide (Fig. 1*C*), whereas cytosolic phosphoglycerate kinase (PGK), a loading control, is constant in all samples.

**ER-to-Golgi Transport Delay and Suppression at High Temperature.** Because *orm1*Δ *orm2*Δ cells seem to accumulate misfolded protein, intracellular transport was assayed by pulse-chase experiments. Transport of each of three cargos, Gas1, carboxypeptidase Y (CPY), and proteinase A, seem delayed in *orm1*Δ *orm2*Δ cells by comparison with wild-type cells (Fig. S2). Delayed appearance of mature Gas1 and persistence of the P1 form of CPY suggest slowed ER-to-Golgi transport.

Because HSR can participate in alleviating ER stress (11), we tested whether mild heat shock conditions can improve resistance of *orm1*Δ *orm2*Δ cells to tunicamycin. As shown in Fig. S3*A*, incubation at 39 °C results in improved growth of *orm1*Δ *orm2*Δ cells in the presence of tunicamycin. Incubation at 37 °C is also sufficient to suppress tunicamycin sensitivity. To test whether suppression is mediated by HSR, a constitutively active Hsf1 was used to induce HSR without shift to high temperature (12). Surprisingly, tunicamycin sensitivity of *orm1*Δ *orm2*Δ cells is not affected by constitutively active Hsf1 (Fig. S3*A*). By contrast, as a positive control, constitutively active Hsf1 rescues the tunicamycin sensitivity of *ire1*Δ cells deficient in UPR (Fig. S3*A*) (11). Thus, the suppressing effect of high temperature seems to be independent of transcriptional induction of heat shock proteins.

Slowed ER-to-Golgi transport of newly synthesized CPY observed at 25 °C is no longer apparent in *orm1*Δ *orm2*Δ cells at 37 °C (Fig. S3*B*). Thus, high temperature suppresses tunicamycin sensitivity as well as the vesicular transport defect of *orm1*Δ *orm2*Δ cells.

**Regulation of Sphingolipid Synthesis.** Several high-throughput studies suggest genetic and physical interactions between the Orm proteins and components of the sphingolipid biosynthesis pathways (13, 14). We used genetic and biochemical approaches to test whether sphingolipid synthesis is affected in *orm1*Δ *orm2*Δ cells. Sphingolipid synthesis involves production of ceramide via acylation of a long chain base with a very long chain fatty acid in the ER. The first committed step is catalyzed by the SPT activity of Lcb1 and Lcb2 and is inhibited by myriocin (ref. 3; pathway diagram in Fig. S4). Because SPT activity is an essential activity, a high dose of myriocin kills cells. As shown in Fig. 2*A*, a sublethal dose of myriocin was used to reduce sphingolipid synthesis without impairing cell growth. Low-dose myriocin suppresses cold sensitivity and tunicamycin sensitivity in *orm1*Δ *orm2*Δ cells (Fig. 2*A*). Low-dose myriocin also rescues slow ER-to-Golgi transport in *orm1*Δ *orm2*Δ cells (Fig. 2*B*).

A mutant in the sphingolipid pathway, *lcb3*, was suggested to interact genetically with the Orm proteins by high-throughput assay (13). *LCB3* is an ER-localized phosphatase that dephosphorylates exogenously imported long chain base phosphates, an activity necessary for incorporation of exogenous long chain bases into sphingolipids (Fig. S4) (3). An *lcb3*Δ *orm1*Δ *orm2*Δ triple mutant is resistant to tunicamycin by comparison with *orm1*Δ *orm2*Δ double mutants; growth of *lcb3*Δ cells in the presence of tunicamycin is similar to that of wild-type cells (Fig. S5*A*). Like myriocin, *lcb3*Δ also restores rapid ER export of CPY in *orm1*Δ *orm2*Δ cells, similar to wild-type cells (Fig. S5*B*). Finally, as shown in Fig. S5*C*, a low basal level of UPR is restored in the triple mutant, indicating that inhibition of the sphingolipid synthesis pathway alleviates multiple phenotypes of *orm1*Δ *orm2*Δ cells.

Suppression by myriocin or *lcb3*Δ suggests perturbation of the sphingolipid synthesis pathway in *orm1*Δ *orm2*Δ cells. Consistent

with this idea, mass spectrometry shows a 4.8-fold increase in accumulation of the long chain base phytosphingosine (PHS) in *orm1*Δ *orm2*Δ cells compared with wild-type cells (Fig. 3*A*). Increased de novo synthesis seems to account for increased PHS because PHS returns to wild-type levels after addition of myriocin to *orm1*Δ *orm2*Δ cells for 2 h (Fig. 3*A*). Fig. 3*B* shows that *orm1*Δ *orm2*Δ cell growth is sensitive to exogenously added PHS. [To exclude effects of tryptophan and uracil auxotrophies in exacerbating PHS sensitivity (15), *TRP*$^+$ strains bearing *URA3*-marked plasmids were examined.] Because the long chain base transporter Rsb1 promotes efflux of long chain bases from the cell (16), we tested the effect of *RSB1* overexpression on PHS sensitivity of *orm1*Δ *orm2*Δ cells. Fig. 3*B* shows that high-copy *RSB1* rescues impaired growth of *orm1*Δ *orm2*Δ cells in the presence of PHS. Western blot shows increased HA-Rsb1 protein level in *orm1*Δ *orm2*Δ cells (Fig. 3*C*), consistent with a possible compensatory response to accumulated long chain base.

A genetic approach was taken to understanding Orm protein function. Selection for suppressors of *orm1*Δ *orm2*Δ cells after random insertional mutagenesis yielded disruption in the *LCB1* promoter region. This provided motivation for using coimmunoprecipitation (co-IP) assay to detect whether there is physical interaction between the Orm proteins and Lcb1. Fig. 4*A* shows that HA-Lcb1 was observed in pull-downs of Orm1-TAP and Orm2-TAP. Negative controls show that IgG-Sepharose does not precipitate HA-Lcb1 in the absence of TAP-tagged Orm2 (wild type) (Fig. 4*A*), and pull-down of Orm2-TAP does not include myc-tagged Erg11, an ER membrane protein (17) (Fig. 4*B*). SPT is composed of a heterodimer of Lcb1 and Lcb2 (18). HA-Lcb2 is also present in an Orm2-TAP pull-down (Fig. 4*C*). However, co-IP of Orm2-TAP ad HA-Lcb1 occurs with similar efficiency in the presence or absence of Lcb2 (Fig. 4*D*). These results suggest the possibility that Orm2 interaction with SPT occurs via Lcb1. Physical association of Orm1 and Orm2 with both SPT subunits, together with PHS accumulation and suppression of *orm1*Δ *orm2*Δ cells by myriocin, support a role for the Orm proteins in negative regulation of SPT.

To ask whether flux through the sphingolipid synthesis pathway is increased in *orm1*Δ *orm2*Δ cells, ceramide levels were measured in wild-type and mutant cells. Surprisingly, ceramide is decreased ≈2-fold in *orm1*Δ *orm2*Δ cells (Fig. S6*A*). Ceramide is produced upon linkage of long chain base to a very long chain fatty acyl CoA produced via a series of reactions to elongate fatty acyl coA (Fig. S4*A*) (3). Very long chain fatty acid levels, like PHS, are also increased in *orm1*Δ *orm2*Δ cells (Fig. S6*B*). Although further work is necessary to determine whether de novo ceramide synthesis is impaired, decreased ceramide suggests that flux in the later sphingolipid pathway is not increased in *orm1*Δ *orm2*Δ cells.

**Inositol Auxotrophy in *orm1*Δ *orm2*Δ Cells.** Inositol auxotrophy accompanies perturbed sphingolipid homeostasis in *orm1*Δ *orm2*Δ cells (Fig. 5). It is well established that expression of *INO1*, encoding inositol 1-phosphate synthase (and other $UAS_{INO}$ genes encoding phospholipid biosynthesis proteins), is regulated by transcriptional activators, Ino2 and Ino4, and a repressor, Opi1 (4). Fig. 5*A* shows that inositol auxotrophy of *orm1*Δ *orm2*Δ cells is suppressed by *INO1* overexpression and is dependent on Opi1. Thus, Orm1 and Orm2 may act upstream of transcriptional repression by Opi1. An *INO1-lacZ* reporter was

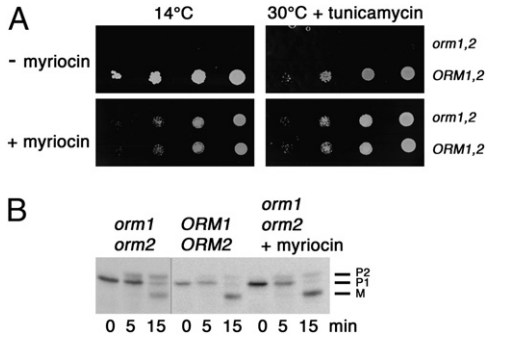


**Fig. 2.** Suppression of *orm*Δ*1 orm2*Δ phenotypes by myriocin. (*A*) Suppression of *orm1*Δ *orm2*Δ cells by low-dose myriocin. Serial dilutions of cells were spotted onto SC medium with myriocin (0.05 μg/mL). Plates with or without tunicamyin (1 μg/mL) and myriocin were incubated at 30 °C for 2 days. To test cold sensitivity, cells were incubated for 5 days at 14 °C. (*B*) Slow ER-to-Golgi transport is suppressed by myriocin. Myriocin (1 μg/mL) was added 3 h before pulse-labeling as described in Fig. S2 legend.

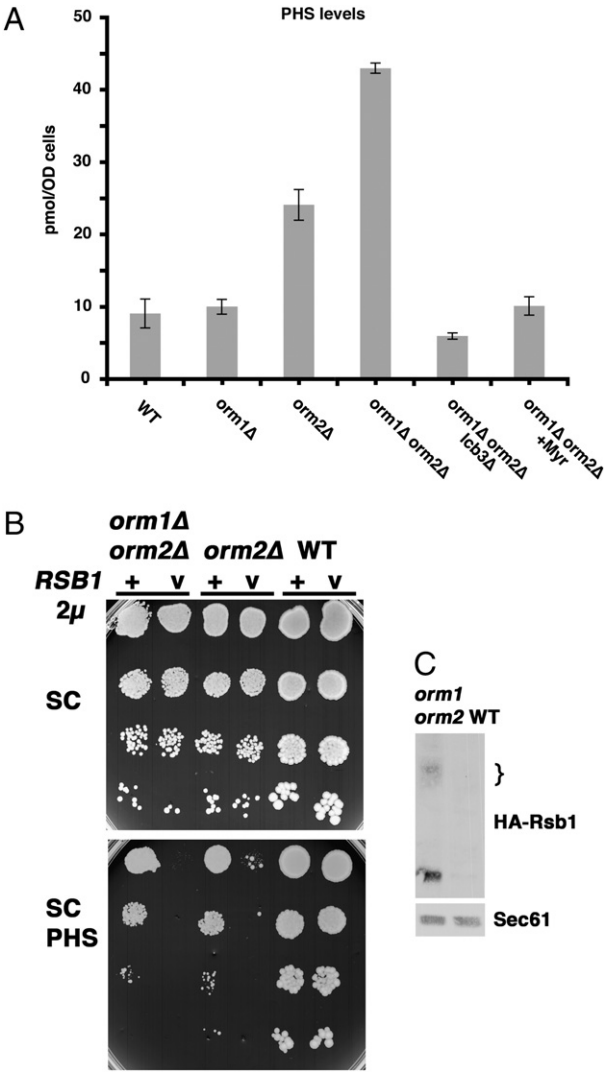


**Fig. 3.** Perturbation in sphingolipid homeostasis in *orm1*Δ *orm2*Δ cells. (*A*) Long chain base was quantitated by mass spectrometry in wild-type and single *orm* mutants, *orm1*Δ *orm2*Δ, and *lcb3*Δ *orm1*Δ *orm2*Δ cells, as described in *Methods*. Myriocin (0.5 μg/mL) was added to *orm1*Δ *orm2*Δ cells for 2 h. (*B*) Hypersensitivity of *orm1*Δ *orm2*Δ cells to exogenous PHS and suppression by high-copy *RSB1*. Cells with vector or *RSB1* on a 2-μ plasmid were serially diluted and spotted on plates with SC medium with or without PHS (10 μM). (*C*) Rsb1 expression is increased in the absence of Orm1 and Orm2. *rsb1*Δ (ACX161-4C) and *rsb1*Δ *orm1*Δ *orm2*Δ (ACX161-4D) cells bearing a centromeric plasmid with *RSB1*-HA were analyzed by Western blot with anti-HA antibody. Brackets indicate glycosylated Rsb1, as described previously (16).

used to confirm that inositol auxotrophy of *orm1*Δ *orm2*Δ cells is due to impaired derepression of *INO1* and other $UAS_{INO}$ genes. Fig. 5*B* shows that *orm2*Δ and *orm1*Δ *orm2*Δ double mutants fail to derepress the reporter in the absence of inositol.

Derepression occurs as Opi1 dissociates from the promoters of *INO1* and other phospholipid synthesis genes and is sequestered at the ER membrane by interaction with the VAMP-associated protein homolog, Scs2 (19). Phosphatidic acid (PA) also stimulates Opi1 tethering at the ER membrane (20). Consistently, inositol auxotrophy of *orm1*Δ *orm2*Δ cells is suppressed by both overexpression of *SCS2* and overexpression of *DGK1*, encoding diacylglycerol kinase, which promotes increased conversion of diacylglycerol to PA (Fig. 5*A*).

Remarkably, myriocin rescues slow growth (Fig. 5*A*) and causes a slight increase in *INO1-lacZ* expression in *orm1*Δ *orm2*Δ cells in the absence of inositol (Fig. 5*B*). Interestingly, when wild-type cells are starved for inositol, myriocin causes decreased *INO1-lacZ* expression, reflecting coordinate regulation of sphingolipid and phospholipid synthesis (21). One possible explanation for the result in wild-type cells comes from the observation that long chain base inhibits PA phosphatase (Pah1) activity (21); decreased long chain base levels in the presence of myriocin could result in increased Pah1 activity, decreased PA levels, and impaired derepression of $UAS_{INO}$ genes.

## Discussion

Orm1 and Orm2 are yeast members of a conserved family of ER membrane proteins whose physiologic significance is emphasized by linkage of a human family member to asthma susceptibility (6). Family members are likely to have conserved function because human *ORMDL3* complements the yeast *orm1 orm2* mutant (5). The two yeast genes also have overlapping function because overexpression of either *ORM1* or *ORM2* can suppress phenotypes of the double-mutant cells.

A major finding in this study is that the Orm proteins regulate sphingolipid homeostasis. Orm1 and Orm2 physically interact with Lcb1 and Lcb2 (Fig. 4). Because SPT activity requires association of Lcb1 and Lcb2 (18), our results suggest that the Orm proteins work together to regulate SPT activity. PHS accumulation in *orm1*Δ *orm2*Δ cells is abolished by myriocin addition (Fig. 3*A*), further supporting dysregulation of SPT activity and increased de novo sphingolipid synthesis. Consistently, the long chain base transporter Rsb1 is up-regulated in *orm1*Δ *orm2*Δ cells, and Rsb1 overexpression rescues cell sensitivity to exogenous PHS (Fig. 3 *B* and *C*). Significantly, *LCB3* mutation suppresses PHS accumulation (Fig. 3*A*), and inhibition of sphingolipid synthesis (by myriocin addition or *LCB3* mutation) suppresses essentially every phenotype of *orm1*Δ *orm2*Δ cells (Fig. 2 and Fig. S5). Inhibitors or mutations impairing steps in sphingolipid synthesis downstream of Lcb3 have weaker suppressing effects (Fig. S7). These results are consistent with a hyperactive SPT in *orm1*Δ *orm2*Δ cells that is compensated by inhibition of the pathway.

PHS accumulation does not reflect increased flux through the sphingolipid pathway because ceramide levels are decreased (rather than increased) in *orm1*Δ *orm2*Δ cells (Fig. S6*A*). A

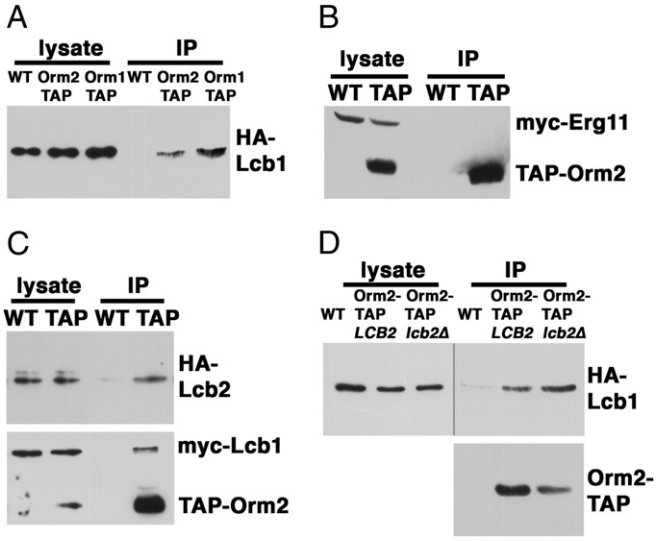


**Fig. 4.** Co-IP of Orm proteins and SPT subunits. Exponentially growing cells in SC medium were harvested, and lysate was prepared and incubated with IgG-Sepharose under nondenaturing conditions. IPs were analyzed by Western blot with anti-rabbit to detect Orm2-TAP, and anti-HA or anti-myc antibodies. (*A*) Lysate was prepared from wild-type (untagged *ORM*s) and cells with chromosomal TAP-tagged *ORM1* (SHY53) and *ORM2-TAP* bearing a centromeric plasmid with HA-*LCB1*. Pull-downs were analyzed by Western blot with anti-HA antibody to detect Lcb1. (*B*) Lysate was prepared from wild-type and TAP-*ORM2* cells bearing a centromeric plasmid with myc-*ERG11*. After Orm2-TAP pull-down, anti-myc antibody was used to detect Erg11 as a negative control, and Orm2 was detected by rabbit secondary antibody. (*C*) Lysate was prepared from wild-type and *ORM2-TAP* cells bearing two plasmids with myc-tagged *LCB1* and HA-*LCB2*. After Orm2-TAP pull-down, Western blots with anti-HA and anti-myc were used to detect HA-Lcb2 and myc-Lcb1, respectively. Efficiency of co-IP is similar for HA-Lcb2 and myc-Lcb1. (*D*) Lysate was prepared from wild-type, *ORM2-TAP*, and *ORM2-TAP lcb2*Δ (SHY54) bearing a centromeric plasmid with HA-tagged *LCB1*. After Orm2-TAP pull-down, anti-HA antibody was used to detect HA-Lcb1.

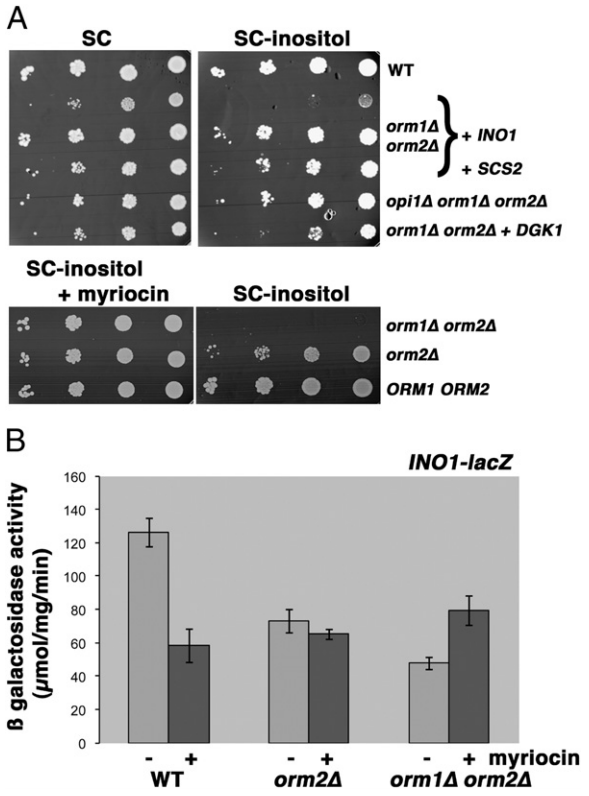


**Fig. 5.** Inositol auxotrophy of *orm1*Δ *orm2*Δ cells. (*A*) Ino$^-$ phenotype of *orm1*Δ *orm2*Δ cells is suppressed by overexpression of *INO1*, *DGK1*, and *SCS2*. Cells were serially diluted and spotted onto plates with SC medium with or without inositol at 30 °C. (*B*) Induction of *INO1-lacZ* is impaired in *orm1*Δ *orm2*Δ cells. Wild-type (HXX1-2A), *orm2*Δ (HXX1-7A), and *orm1*Δ *orm2*Δ (HXX1- 7D) cells bearing an *INO1-lacZ* reporter were grown at 25 °C in SC-uracil medium. Cells were washed and resuspended in fresh medium without inositol with or without myriocin (0.5 μg/mL) for 3 h. Lysate was prepared for β-galactosidase activity measurement. Assays were performed in duplicate on at least three independent colonies.

decrease in ceramide synthesis in *orm1*Δ *orm2*Δ cells is a simple hypothesis that accounts for accumulation of both precursors, very long chain fatty acid (Fig. S6*B*) and PHS. Nevertheless, further work is necessary to determine whether decreased ceramide is indeed due to impaired de novo ceramide synthesis. It is also unclear whether decreased ceramide is a direct consequence of loss of Orm1 and Orm2. The contribution of decreased ceramide to the pleiotropic phenotypes of *orm1*Δ *orm2*Δ cells is uncertain because the ceramide synthase inhibitor fumonisin has a mild suppressing effect (Fig. S7). Consistent with perturbation of long chain base and ceramide levels, *orm1*Δ *orm2*Δ cells have impaired HSR and cell wall stress response (Fig. S1); long chain base and ceramide play signaling roles for these signal transduction pathways (22, 23).

Like myriocin, heat is also an effective suppressor of *orm1*Δ *orm2*Δ phenotypes (Fig. S3). Heat is known to up-regulate de novo sphingolipid synthesis (24). Therefore, heat-mediated suppression may occur via a mechanism distinct from myriocin. One possibility is that a heat-mediated increase in membrane fluidity might counteract increased sphingolipid synthesis in *orm1*Δ *orm2*Δ cells.

Disrupted phospholipid homeostasis is evidenced by inositol auxotrophy of *orm1*Δ *orm2*Δ cells (Fig. 5). Sphingolipid and phospholipid synthesis pathways intersect as they share common metabolites: phosphatidylinositol; diacylglycerol is used for synthesis of phosphatidylethanolamine (PE), phosphatidylcholine, and triacylglycerols; long chain base phosphates are converted for use in PE synthesis (4, 21) (Fig. S4). Moreover, it has been suggested that sphingolipid metabolism is regulated by phosphoinositide signaling pathways (25, 26). *ORM2* itself represents a link between sphingolipid and phospholipid pathways: it is an Ino2-Ino4 target gene, repressed by inositol (27). Myriocin-mediated rescue of inositol auxotrophy in *orm1*Δ *orm2*Δ cells supports a model in which the other lipid pathways are affected by disrupted sphingolipid homeostasis. More work is necessary to understand impaired derepression of phospholipid synthesis genes in *orm1*Δ *orm2*Δ cells. A recent report suggests that lipid packing within membranes can regulate derepression of phospholipid gene transcription (28).

Because membrane biogenesis requires orchestration of lipid and protein synthesis at the ER, loss of Orm1 and Orm2 results in multiple effects on protein quality control: constitutive activation of UPR, sensitivity to agents that elicit cell stress, and slowed ER-to-Golgi transport (Figs. 1 and 2). In mammalian cells, RNA interference to knock down all three members of the ORMDL family results in impaired maturation of nicastrin, a component of the gamma secretase complex, which is involved in proteolytic processing of substrates such as amyloid precursor protein, Notch, and cadherins (29). Maturation of nicastrin is dependent on trafficking from ER to Golgi, and mature nicastrin in post-ER compartments is then able to assemble with presenilins to form active enzyme complexes. The phenotype in mammalian cells is consistent with defective protein quality control in the absence of Orm1 and Orm2 in yeast.

We propose that slow ER-to-Golgi transport is a consequence of disturbed lipid homeostasis in *orm1*Δ *orm2*Δ cells. Several studies have suggested that vesicular transport is dependent on ceramide (30, 31), and ER export is facilitated by PA and sterols (32, 33). Conversely, defective ER-to-Golgi transport leads to defective phospholipid synthesis (34). In *orm1*Δ *orm2*Δ cells, it is possible that imbalanced lipids are also responsible for constitutive UPR. Accumulation of saturated fatty acids and ergosterol in the ER has been reported to induce UPR (35). UPR is induced in inositol auxotrophs (36) as well as in wild-type cells starved for inositol (37). UPR-deficient mutants such as *ire1*Δ cells are inositol auxotrophs (37), although the coregulatory mechanism remains unclear. UPR has also been reported to regulate expression of a number of genes functioning in lipid metabolism, including fatty acid and sterol metabolism, and phospholipid and sphingolipid synthesis (2, 38). Indeed, *ORM2* itself is also under UPR purview (Fig. 1*C*). Because many membrane proteins require a proper lipid environment to achieve their proper conformation (39, 40), it seems probable that disrupted lipid balance can increase protein misfolding.

The central role played by Orm1 and Orm2 in membrane biogenesis at the ER suggests a similar role for other members of the ORMDL protein family. Our analysis of pleiotropic phenotypes of *orm1*Δ*orm2*Δ cells has revealed complex interrelationships between different lipid homeostatic mechanisms as well as coregulation of protein quality control with lipid status. Further work to dissect these complicated relationships should increase our understanding of how lipid and protein synthesis are coordinated during membrane biogenesis.

## Methods

**Strains and Media.** Standard yeast media were used (41). Synthetic media was supplemented with 200 μM myoinositol. Strains used in this study are described in *SI Methods*. SHY54, an *lcb2*Δ strain, was grown in synthetic complete (SC) medium containing 15 μM PHS and 0.1% tergitol, as described previously (42).

**Molecular Biology.** Plasmids used in this study are described in *SI Methods*.

**Metabolic Labeling, IP, and Western Blot.** Metabolic protein labeling was done as previously described (43). Briefly, cells were grown in minimal medium to midlog phase and then resuspended at 1 $OD_{600}$/mL before pulse-labeling with Expre$^{35}$S$^{35}$S (Perkin-Elmer) for 5 min at room temperature. Cells were chased with an equal volume of SC medium plus 20 mM methionine and cysteine. Chase was terminated by addition of Na azide (10 mM). Lysate was prepared by vortexing cells with glass beads (43). For IP, lysates were normalized to acid-precipitable cpm, and solubilized in 1% SDS, boiled, and diluted to 0.1% SDS with radioimmunoprecipitation assay buffer minus SDS. IPs with anti-CPY (Invitrogen) were analyzed by SDS/PAGE and fluorography.

To isolate TAP-tagged Orm2, lysate was prepared by vortexing cells with glass beads in PBS buffer with 200 mM sorbitol, 1 mM $MgCl_2$, and 0.1% Tween 20 in the presence of a protease inhibitor mixture. Lysate was normalized to protein content by Bradford assay and incubated overnight at 4 °C with IgG-Sepharose (GE Healthcare). Beads were washed eight times with buffer containing 0.8% Tween 20 and analyzed by Western blot. Antibody binding to Western blots was visualized by peroxidase-conjugated secondary antibody, followed by a chemiluminescence detection system. Anti-Sec61 (Randy Schekman, University of California, Berkeley) and anti-Kar2 (Mark Rose, Princeton University) are gifts. Anti-PGK, anti-HA monoclonal, and anti-myc polyclonal antibodies were purchased from Invitrogen, Covance, and Santa Cruz Biotechnology, respectively.

**Lipid Analysis.** For mass spectrometry, lipids were extracted with $CHCl_3$:MeOH (17:1; per vol) from 10 OD of cells containing 125 pmol C17 dihydrosphingosine, 170 pmol of C18-ceramide, or 150 pmol of free C22:1 fatty acids as internal standards (44). Long chain bases were analyzed in the positive ion mode and C26 and Cer in the negative ion mode on a Bruker Esquire HCT ion trap mass spectrometer (ESI) at a flow rate of 180 μL/h and a capillary tension of −250 V. Ion fragmentation was induced by argon as collision gas at a pressure of 8 mbar. $[M+H]^+$ ions of phytosphingosine and $[M-H]^-$ ions of C26 and ceramide species were quantified relative to the internal standards.

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