

Regulation of sphingolipid synthesis via Orm1 and Orm2 in yeast

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Summary

Sphingolipids are critical components of membranes and sphingolipid metabolites also serve as signaling molecules. Yeast Orm1 and Orm2 belong to a conserved family of ER membrane proteins that regulate serine palmitoyltransferase, catalyzing the first and rate-limiting step in sphingolipid synthesis. We now show that sphingolipid synthesis via Orm1 is a target of TOR signaling which regulates cell growth in response to nutritional signals. Orm1 phosphorylation is dependent on the Tap42-phosphatase complex which acts downstream of TOR protein kinase complex 1; in temperature-sensitive *tap42-11* cells, impaired Orm1 phosphorylation occurs concomitantly with reduced sphingolipid synthesis. A second mechanism regulating sphingolipid synthesis is via controlling Orm2 protein level. Orm2 protein level responds to ER stress conditions, increasing when cells are treated with tunicamycin or DTT, agents that induce the unfolded protein response (UPR). The sphingolipid intermediates, long chain base and ceramide, are decreased when *ORM2* is overexpressed, suggesting sphingolipid synthesis is repressed under ER stress conditions. Finally, in the absence of the Orms, the UPR is constitutively activated. Lipid dysregulation in the absence of the Orms may signal to the ER from the plasma membrane as UPR activation is dependent on a cell surface sensor and the MAPK cell wall integrity pathway. Thus, sphingolipid synthesis and the UPR are coordinately regulated.

Introduction

Membrane biogenesis during cell growth and proliferation involves coordination of protein and lipid synthesis. In response to nutritional conditions, protein synthesis is modulated via multiple signaling pathways (Smets et al., 2010). One of these is TOR (target of rapamycin), a conserved protein kinase complex that regulates growth in response to nutrients and stresses. The unfolded protein response (UPR) pathway also plays a role in membrane biogenesis by adjusting the capacity of the endoplasmic reticulum (ER) to handle the load of newly synthesized proteins (Cox et al., 1997; Ron and Walter, 2007; Rutkowski and Hegde, 2010). The ER also serves as the initiation site for synthesis of the major lipid components of membranes. Transcriptional regulation serves as a major mechanism for controlling phospholipid and sterol synthesis (Nohturfft and Zhang, 2009). Insight into regulation of sphingolipid synthesis has come from recent discovery of the conserved ORMDL family of ER membrane proteins (Breslow et al., 2010; Han et al., 2010).

Sphingolipids are critical structural components of membranes, contributing to key physical properties (Breslow and Weissman, 2010). Sphingolipid metabolites also have important signaling functions. The ORMDL proteins regulate sphingolipid synthesis by physically associating with serine palmitoyltransferase (SPT) which catalyzes the first and rate-limiting step of sphingolipid synthesis. SPT mediates long chain base production from the condensation of serine and palmitoyl CoA (Funato et al., 2002). Ceramide is then generated upon addition of a second long chain fatty acid to long chain base. Mature sphingolipids are made in the Golgi upon transport of ceramide from the ER. The ORMDL proteins repress SPT activity; in the absence of ORMDL regulation, SPT activity becomes hyperactive and long chain base accumulates (Breslow et al., 2010; Han et al., 2010). In yeast, SPT activity is regulated by Orm1 and Orm2, and phosphorylation of the Orm proteins adjust SPT activity to maintain sphingolipid homeostasis (Breslow et al., 2010). In *orm1Δ orm2Δ* cells, dysregulation of sphingolipid synthesis results in pleiotropic phenotypes, including impaired growth and constitutive activation of the unfolded stress response (UPR) (Han et al., 2010).

As a first step to understanding how sphingolipid synthesis is regulated in response to growth conditions, we have identified the TOR signaling pathway as one regulatory component controlling sphingolipid synthesis via Orm1. In this study, we show that Orm1 phosphorylation and sphingolipid synthesis is dependent on the Sit4-Tap42 complex, a downstream target of the rapamycin-sensitive TOR protein kinase complex, TORC1. Another mechanism to regulate sphingolipid synthesis is via changing Orm2 protein level. Orm2 protein level is increased by agents that cause ER stress, and sphingolipid synthesis is repressed upon *ORM2* overexpression.

Results

Orm1 phosphorylation and sphingolipid synthesis respond to the TOR signaling pathway

In a recent large-scale proteomic study, Orm1 was identified as a possible target phosphorylated by the TOR signaling pathway (Huber et al., 2009). Phosphorylation of TAP-tagged Orm1 was detected as electrophoretic mobility shifts (Fig. 1). Orm1 is constitutively phosphorylated on multiple residues [(Breslow et al., 2010) & Fig. 3 below]. Thus, under basal conditions, Orm1 phosphorylation appears heterogeneous and this is reflected by Western blot as two or more bands (Fig. 1A, lane 1). When cells are treated with myriocin to inhibit SPT, phosphorylation of Orm1-TAP is increased, visualized as additional bands with decreased electrophoretic mobility (Fig. 1A, lane 2). To assess whether Orm1 is a possible target of the TOR pathway, Orm1-TAP was examined after treating cells with rapamycin to inhibit TORC1 activity. Western blot for Orm1-TAP revealed additional bands with decreased electrophoretic mobility after rapamycin addition for 20 min. (Fig. 1A, lane 3), confirming a dependence of Orm1 phosphorylation on TORC1. Rapamycin stimulates Orm1 phosphorylation, but was not observed to affect Orm2 phosphorylation (Fig. 1B).

The Tap42-Sit4-PP2A protein phosphatase complex acts downstream of TORC1 to regulate growth in response to cellular nutrient status (Smets et al., 2010). To test whether this branch of the TOR signaling pathway participates in Orm regulation, electrophoretic mobility of Orm1-TAP and Orm2-TAP was assessed in *sit4Δ* cells. As shown in Fig. 1A, basal phosphorylation of Orm1 appears decreased in *sit4Δ* cells, and response to myriocin and rapamycin is abrogated; by contrast, basal phosphorylation of Orm2 and response to myriocin are unaffected in *sit4Δ* cells.

In *sit4Δ* cells, decreased Orm1 phosphorylation correlates with decreased long chain base and ceramide levels, measured by mass spectrometry (Fig. 1C). A decreased level of these sphingolipid intermediates is consistent with a model in which SPT activity is repressed by dephosphorylated Orm1.

Orm1 phosphorylation and sphingolipid synthesis in tap42-11 cells

Because numerous pleiotropic phenotypes are associated with *sit4Δ* cells, a temperature-sensitive *tap42-11* mutant was used to inactivate TOR signaling. The *tap42-11* allele confers rapamycin resistance at 25°C and lethality at 37°C (Cherkasova and Hinnebusch, 2003). In Fig. 2A, *TAP42⁺* and *tap42-11* cells were grown at 30°C and shifted to 37°C in the presence or absence of myriocin for 1h. A comparison of Orm1-TAP electrophoretic mobility is shown in Fig. 2A: stimulated phosphorylation in response to rapamycin is barely detectable in *tap42-11* cells at 30°C, and undetectable at 37°C.

To improve resolution of electrophoretic mobility shifts, Orm1 protein was tagged with a single HA epitope (Fig. 2B). In wild-type cells, a sizeable electrophoretic mobility shift was observed upon increased phosphorylation of HA-Orm1 stimulated by myriocin and rapamycin (Fig. 2B, left panel, arrow). All bands reflect different degrees of phosphorylation as they collapse to a single band upon alkaline phosphatase treatment (Fig. 3). Phosphorylation of HA-Orm1 in *TAP42⁺* and *tap42-11* cells was quantitated by scanning Western blots and plotting maximally phosphorylated HA-Orm1 (topmost band) as a percent of total HA-Orm1 (Fig. 2B, right panel). By comparison with wild-type cells, rapamycin-induced phosphorylation in *tap42-11* cells is reduced at 30°C (Fig. 2B, lane 3). At 37°C, response to rapamycin in *tap42-11* cells is undetectable (Fig. 2B, bottom panel, compare lanes 3 & 6). At 37°C, basal and myriocin-stimulated phosphorylation of Orm1 are significantly reduced in *tap42-11* cells (Fig. 2B, bottom panel, lanes 4 and 5).

Ceramide levels were compared in wild-type and *tap42-11* cells by mass spectrometry. Cells were grown at 24°C and then shifted to either 30°C or 37°C for 2h before lipid analysis. In *tap42-11* cells, shift to 37°C results in decreased ceramide levels (Fig. 2C). Upon Tap42 inactivation, the temporal correspondence between diminished levels of ceramide, and reduced Orm1 phosphorylation (Figs. 2A & B) suggests that inhibition of sphingolipid synthesis is a consequence of Orm1 dephosphorylation.

The Ser/Thr protein kinase Npr1 is controlled by TOR and Tap42, and responds to nutrient conditions (Schmidt et al., 1998). In *npr1Δ* cells, Orm1 phosphorylation is abrogated in

response to rapamycin (Fig. 2D, compare lanes 3 & 6, arrow) and Orm1 phosphorylation in response to myriocin is impaired (compare lanes 2 & 5).

Orm1 phosphorylation responds to Orm2 status

Cooperation between Orm1 and Orm2 has been suggested by genetic evidence showing that *orm1Δ orm2Δ* phenotypes are suppressed by either high copy *ORM1* or high copy *ORM2* (Han et al., 2010). Physical interaction between Orm1 and Orm2 further supports a cooperative relationship (Breslow et al., 2010; Han et al., 2010). Fig. 3A shows that Orm1 phosphorylation responds to Orm2 status. Under basal conditions in *ORM2⁺* cells where HA-Orm1 is the sole copy of Orm1, HA-Orm1 is heterogeneously phosphorylated with lesser phosphorylated forms predominating (Fig. 3A, lane 7). In *orm2Δ* cells, HA-Orm1 is dephosphorylated with an electrophoretic mobility like that from *ORM2⁺* cells treated with alkaline phosphatase (Fig. 3A, compare lanes 8 & 9). In *orm2Δ* cells, myriocin addition results in increased HA-Orm1 phosphorylation (lane 3), suggesting SPT activity is a major influence on Orm1 phosphorylation state. HA-Orm1 in *orm2Δ* cells appears fully dephosphorylated as no effect on its mobility was detected after treatment with alkaline phosphatase (compare lanes 11 & 12, arrow). However, in *orm2Δ* cells, HA-Orm1 phosphorylation is increased in response to myriocin addition (lane 3), suggesting SPT activity is a major influence on Orm1 phosphorylation state. When Orm2 is overexpressed, HA-Orm1 phosphorylation is constitutively increased (lane 4, asterisk). In cells with high copy *ORM2*, growth on plates with low dose myriocin is compromised (Fig. 3A, bottom panel), consistent with increased repression of SPT activity upon Orm2 overexpression. Together, these results support a model in which Orm1 phosphorylation offsets fluctuations in Orm2 levels to maintain homeostatic control of SPT.

Sac1, a phosphoinositide phosphatase, is another component of the Orm protein complex named SPOTS (containing serine palmitoyltransferase, Orm1, Orm2, Tsc3, Sac1) (Breslow et al., 2010). Fig. 3B shows that Orm1 phosphorylation is constitutively increased in *sac1Δ* cells (lane 3). [Because there is a chromosomal copy of *ORM1* in *sac1Δ* cells in addition to HA-Orm1, two controls are shown in Fig. 3B. Basal phosphorylation of HA-Orm1 is

increased in cells with chromosomal *ORM1* (lane 2) by comparison with cells in which HA-Orm1 is the sole copy of *ORM1* (lane 1).] Phosphatidylinositol is a component of complex sphingolipids in yeast, and it has been reported that sphingolipid metabolism is modulated by phosphatidylinositol levels regulated by Sac1 (Brice et al., 2009). Like response to myriocin, increased phosphorylation of Orm1 in *sac1Δ* cells may also reflect response to inhibited flux through the sphingolipid synthesis pathway.

Orm1 phosphorylation in response to myriocin-mediated inhibition of SPT occurs at multiple residues at its amino terminus (Breslow et al., 2010). Ser32 and Ser34 of Orm1 were identified in a large scale assay as residues phosphorylated in response to rapamycin (Huber et al., 2009). Fig. 3C examines phosphorylation of an Orm1 mutant with 5 Ser-Ala changes (S29A, S32A, S34A, S35A, S36A). Constitutive phosphorylation of mutant Orm1 is reduced compared to that of wild-type Orm1 (Fig. 3C, lanes 5 & 8). Stimulated phosphorylation of Orm1 in response to rapamycin is abrogated (lane 11). Response to myriocin is impaired as mobility of mutant Orm1 shifts up slightly (compare lane 2 with lane 3) but the maximally phosphorylated band is not present (lane 1, arrow).

Co-immunoprecipitation of Orm1 and the ceramide synthase component, Lac1

In *orm1Δ orm2Δ* cells, ceramide levels are decreased even as SPT activity and long chain base levels are increased (Han et al., 2010). We tested possibility that ceramide synthase activity is regulated by the Orm proteins independently of SPT. Ceramide synthase is comprised of three ER membrane proteins, Lac1, Lag1 and Lip1 (Vallee and Riezman, 2005). To detect physical association with Orm1-TAP, an HA-tagged Lac1 construct was used in pulldown experiments. Fig. 4A shows the presence of HA-tagged Lac1 in an Orm1-TAP pull-down under nondenaturing conditions. HA-Lac1 is also associated with Orm2-TAP (Fig. 4B). Association of Orm1 with Lac1 is independent of Lag1 (Fig. 4A, right panel). Orm1 association with HA-Lac1 also appears independent of Orm1 phosphorylation state as association remains intact after addition of myriocin or upon *ORM2* overexpression when Orm1 phosphorylation is increased. Orm1-TAP interaction with HA-Lac1 is unaffected in *orm2Δ* cells when Orm1 is dephosphorylated (Fig. 4A, left panel). Physical

association with Lac1 suggests a more complex involvement of Orm1 in regulating the sphingolipid synthesis pathway.

Sphingolipid synthesis and ER stress

Because Orm2 protein level is increased by agents that increase ER stress (Han et al., 2010; Hjelmqvist et al., 2002), we examined whether changing Orm2 protein level represents another mechanism for regulating sphingolipid synthesis. *ORM2* was expressed from a high copy plasmid, and sphingolipid intermediates were measured by mass spectrometry. Fig. 5A shows that high copy Orm2 expression results in decreased long chain base and ceramide levels; Orm1 overexpression also induces decreased ceramide. These findings predict that sphingolipid synthesis is decreased by Orm2 protein induced during ER stress response.

To examine response of Orm1 to ER stress, cells were treated with tunicamycin, inhibitor of N-linked glycosylation inhibitor, or the reducing agent DTT. As shown in Fig. 5B, Orm1 phosphorylation is stimulated by tunicamycin and DTT treatments. Strikingly, in the absence of Orm2, Orm1 phosphorylation does not respond to tunicamycin or DTT (Fig. 5B, arrow). In the absence of Orm2, Orm1 phosphorylation still increases in response to myriocin addition (Fig. 5B). These results suggest that Orm1 phosphorylation is increased as a compensatory response to increased Orm2 levels during ER stress, similar to increased Orm1 phosphorylation upon *ORM2* overexpression (Fig. 3A).

*Constitutive UPR in *orm1Δ orm2Δ* cells is signaled via the plasma membrane*

ER stress-inducing agents affect sphingolipid synthesis by increasing Orm2 protein levels (Han et al., 2010). Conversely, in the absence of Orm1 and Orm2, UPR is constitutively activated (Han et al., 2010). Recent reports have suggested that a MAPK signaling cascade is activated by disruption of sphingolipid homeostasis (Jesch et al., 2010). Cross-talk between MAPK signaling and UPR pathways has been suggested as activation of UPR can occur in

response to perturbation at the plasma membrane (Krysan, 2009). To assay signaling of constitutive UPR in *orm1Δ orm2Δ* cells, we used a UPR-lacZ reporter. By definition, the ER sensor Ire1 mediates UPR induced by tunicamycin in both wild-type and *orm1Δ orm2Δ* cells, and constitutive UPR in *orm1Δ orm2Δ* cells is also dependent on Ire1 (Fig. 6). Remarkably, signaling of constitutive UPR in *orm1Δ orm2Δ* cells also requires the MAP kinase Slt2/Mpk1 (Fig. 6). Activation of UPR in response to tunicamycin addition is not impaired in *slt2Δ orm1Δ orm2Δ* cells.

The MAP kinase signaling cascade plays a well-established role in maintaining cell wall integrity (CWI) via plasma membrane-localized sensors such as Mid2 (Levin, 2005). To test the possibility that UPR in the absence of Orm1 and Orm2 is signaled from the plasma membrane, UPR-lacZ activity was assayed in *mid2Δ orm1Δ orm2Δ* cells. Fig. 6 shows signaling of constitutive UPR in *orm1Δ orm2Δ* cells is prevented in *mid2Δ* cells. Dependence on Mid2 suggests that signaling occurs from the plasma membrane to initiate the UPR response at the ER. By contrast with Mid2, constitutive UPR in *orm1Δ orm2Δ* cells appears less or insignificantly affected by loss of the CWI sensors Wsc1 and Wsc2 (Fig. 6).

Discussion

A major finding of this study is identification of signaling pathways that regulate sphingolipid synthesis via the Orm proteins. Orm1 phosphorylation is increased upon addition of rapamycin to growth medium to mimic nutrient starvation and inhibit the TOR signaling pathway. Orm1 phosphorylation is dependent on the Tap42-Sit4 protein phosphatase complex which mediates TORC1 signaling to a subset of downstream effectors. The Tap42-Sit4 phosphatase complex controls downstream phosphorylation as well as dephosphorylation events (Huber et al., 2009). At the restrictive temperature in *tap42-11* cells, rapamycin-dependent phosphorylation of Orm1 is abrogated, and myriocin-stimulated phosphorylation is decreased (Fig. 2A & B). In *tap42-11* cells after shift to 37°C, a decrease in ceramide levels accompanies impaired Orm1 phosphorylation (Fig. 2C). These results show that the sphingolipid synthesis is regulated via Orm1 phosphorylation by the TORC1 signaling pathway which coordinates membrane biogenesis with cell growth in response to environmental conditions.

Orm1 phosphorylation responds to the TOR-regulated protein kinase Npr1 (Fig. 2D). Because stimulation of Orm1 phosphorylation by rapamycin is abolished in *npr1Δ* cells, it appears that TOR signaling is conveyed via Npr1. Orm1 residues targeted by the TOR signaling pathway include residues S29, S32, S34-36 because the Orm1-5S-A mutant fails to respond to rapamycin (Fig. 3C). Because Orm1 phosphorylation in response to myriocin is only reduced in *npr1Δ* cells, Orm1 is likely phosphorylated at additional residues by one or more signaling pathway(s) that has not yet been identified. [Previous work suggested Orm1 residues S51-53 are phosphorylated in response to myriocin (Breslow et al., 2010)]. Similarly, the signaling pathway(s) regulating Orm2 is not yet known, but appears independent of TORC1 as Orm2 phosphorylation is rapamycin-independent (Fig. 1B).

Previous work has shown that the Orm proteins respond to the status of the sphingolipid synthesis pathway: increased phosphorylation of both Orm proteins relieves repression of SPT (and restores homeostatic SPT activity when myriocin is added) (Breslow et al., 2010). While both Orm protein respond to SPT activity, Orm1 also responds to Orm2 status, and

Orn1 phosphorylation changes as a compensatory response to increased Orn2 or loss of Orn2 (Fig. 3): Orn1 phosphorylation increases (to derepress SPT activity) when Orn2 is overexpressed, and Orn1 is dephosphorylated in the absence of Orn2 (to increase SPT repression). These results have important physiological implications as Orn2 protein level is adjusted by ER stress conditions (Han et al., 2010). Genetic evidence indicates overlapping functions for Orn1 and Orn2; nevertheless, Orn2 appears to have a prevailing influence on SPT activity as *orn2Δ* cells have a subset of phenotypes associated with *orn1Δ orn2Δ* double mutants whereas *orn1Δ* cells have no observable phenotype (Han et al., 2010).

In the absence of the Orn proteins, SPT activity is derepressed and long chain base accumulates (Breslow et al., 2010; Han et al., 2010). Ceramide levels are decreased in *orn1Δorn2Δ* cells (Han et al., 2010). Impaired de novo ceramide synthesis in *orn1Δ orn2Δ* cells is supported by accumulation of very long fatty acid levels [as ceramide is made by linkage of long chain base with very long chain fatty acyl CoA (Funato et al., 2002)]. We now report physical interaction of Orn1 and Orn2 with the ceramide synthase subunit, Lac1 (Fig. 4). Lac1 was not present in the SPOTS protein complex identified by Orn pulldown (Breslow et al., 2010). Detergent conditions may possibly affect detection of Orn association with Lac1. Physical association between Lac1 and Orn1 remains unaffected by myriocin treatment or *ORM2* overexpression (when Orn1 phosphorylation is increased) or in the absence of Orn2 (when Orn1 is dephosphorylated). Because Lac1 and Lag1 are functionally redundant (Schorling et al., 2001), Orn1-Lac1 association in the absence of Lag1 indicates interaction occurs with a catalytically active subunit. Our results suggest a more direct role for the Orns in regulating ceramide synthesis, although further work is necessary to determine the mechanism by which the Orns may affect ceramide synthase activity.

In the absence of the Orn proteins, UPR is constitutively activated [Fig. 6 & (Han et al., 2010)]. Other reports have linked ER stress and UPR activation with perturbation of lipid homeostasis (Brookheart et al., 2009; Erbay et al., 2009; Pineau et al., 2009; Shechtman et al., 2011). If UPR is solely a response to protein misfolding in the ER, a possible explanation for activated UPR in *orn1Δ orn2Δ* cells is that lipid imbalance causes misfolding of

membrane proteins that have a conformational requirement for lipids. More recent work suggests a larger role for the UPR beyond signaling protein misfolding in the ER to maintaining basal cellular homeostasis by interacting with other signaling pathways (Rutkowski and Hegde, 2010). Such a model could account for our finding that the cell wall integrity MAPK pathway is required for UPR signaling in *orm1Δ orm2Δ* cells (Fig. 6).

Surprisingly, UPR signaling in *orm1Δ orm2Δ* cells requires the plasma membrane protein Mid2 (Fig. 6) which traditionally has a role as a transmembrane sensor of cell wall integrity (Philip and Levin, 2001). One possible explanation for these observations is that UPR is activated in *orm1Δ orm2Δ* cells by a signal originating from the plasma membrane; Mid2 may participate in sensing and reporting lipid homeostasis from the cell surface.

Communication from the plasma membrane to activate the UPR at the ER has previously been proposed (Krysan, 2009), based on the observation that UPR activation in response to the cell wall perturbant calcofluor white is dependent on Mid2 and Slt2 (Scrimale et al., 2009). Similarly, it has been reported recently that ER stress signaling to delay cytokinesis and ER inheritance requires the MAP kinase Slt2 and the cell surface receptor Wsc1 (Babour et al., 2010). Regulation of ER function by plasma membrane events is suggested by another recent paper showing that Sac1 activity at the ER responds to phosphoinositides at the plasma membrane via ER-plasma membrane junctions (Stefan et al., 2010). It is possible that there is similar communication to the ER of sphingolipid status at the cell surface.

The UPR plays a role in lipid homeostasis as it regulates expression of genes functioning in lipid metabolism, including fatty acid and sterol metabolism, and phospholipid and sphingolipid synthesis (Travers et al., 2000). In response to ER stress conditions, Orm1 phosphorylation is increased, but the response requires Orm2 (Fig. 5B), and is mimicked by Orm2 overexpression (Fig. 3A). Thus, it seems likely that increased Orm1 phosphorylation in response tunicamycin and DTT serves as a compensatory response to an increase in Orm2 protein. ER stress promotes increased Orm2 protein (Han et al., 2010; Hjelmqvist et al., 2002). Because sphingolipid intermediates are decreased upon Orm2 overexpression (Fig. 5A), we suggest that increased Orm2 protein levels reflects the mechanism by which sphingolipid synthesis is repressed by ER stress. It has been

proposed that increased phospholipid synthesis and membrane expansion are responses that alleviate ER stress (Schuck et al., 2009). Accordingly, we suggest that during ER stress response, repressed sphingolipid synthesis should change membrane composition, and perhaps the fluidity of the membrane.

Our work has implications for understanding the connection between lipid toxicity and ER stress in human diseases such as obesity and diabetes. There is accruing evidence from mammalian cell culture and animal model studies that ER stress response is triggered by lipid imbalance and chronic ER stress plays a critical detrimental role in lipotoxicity (Brookheart et al., 2009). The best evidence that sphingolipid dysregulation contributes to metabolic disease comes from studies showing that inhibiting sphingolipid synthesis ameliorates disease in animal models (Summers, 2010). In this regard, work in yeast should continue to provide insight into molecular mechanisms regulating sphingolipid homeostasis and response to lipid dysregulation.

Materials and Methods

Strains and plasmids

Yeast strains are isogenic with BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Strains from the deletion collection (Open Biosystems, Huntsville, AL) were confirmed by PCR. HXX1-7D is an *orm1Δ::clonNAT^r orm2Δ::kan^r* mutant (Han et al., 2010). SHY22 is BY4741 with *TRP1* replaced with *HIS3* by marker swap (Cross, 1997). SHY53 has chromosomal *ORM1* tagged with TAP and marked with *TRP1*, generated by transformation of SHY22 with PCR products amplified using pBS1479 as template (Puig et al., 2001). ACY107 is SHY53 with *HIS3* replaced by *URA3* by marker swap. ACX191-2A is *MATα his3Δ1 ORM1::TAP::TRP1*, generated by a cross between BY4742 and SHY53. ACX198-1C is *sit4Δ::kan^r ORM1::TAP::TRP1*, generated by cross between *sit4Δ* and ACX191-2A. CY1077 and CY1078 are *MATa leu2Δ0 lys2Δ0 ura3Δ0 tap42::KAN [TAP42 LEU2 CEN]* and *MATα leu2Δ0 met15Δ0 ura3Δ0 tap42::kanMX4 [tap42-11 LEU2 CEN]*, respectively, from Alan Hinnebusch (NIH, Bethesda, MD) (Cherkasova and Hinnebusch, 2003). ACX216 is a cross between CY1078 and ACY107 to generate *tap42-11 ORM1::TAP::TRP1* (ACX216-4A) and a congenic *TAP42⁺* strain (ACX216-4B). ACX218 is a cross between ACX164-1C (*MATα orm1Δ::clonNAT^r orm2Δ::HIS3*) and *mid2Δ::kan^r*; ACX218-1C is an *orm1Δ orm2Δ mid2Δ* triple mutant. ACX213 is a cross between ACX164-1C and *slt2Δ::kan^r* strains; ACX213-5D is an *orm1Δ orm2Δ slt2Δ* triple mutant. KPX2 and KPX3 are crosses between ACX164-1C and *wsc2Δ::kan^r* and *wsc1Δ::kan^r* strains, respectively; KPX2-2A and KPX3-7B are *orm1Δ orm2Δ wsc2Δ* and *orm1Δ orm2Δ wsc1Δ* triple mutants, respectively. ACX184-2B is *MATα ORM2::TAP::HIS3* from a cross between BY4742 and BY4741 *ORM2::TAP::HIS3* (Open Biosystems). CHY48 is *ORM2::TAP::URA3* generated by transformation of BY4742 with PCR products amplified using pBS1539 as template (Puig et al., 2001). ACX225-1A is an *ORM1::TAP::TRP1 orm2Δ* strain generated by cross between HXX1-7A and SHY53.

pSH16 and pSH17 are *LEU2*-marked 2μ plasmid bearing *ORM1* and *ORM2*, respectively (Han et al., 2010). pSH14 is a *HIS3*-marked centromeric plasmid bearing *ORM1* as a 2 kb

insert. pSH14HA has an HA epitope introduced after the initiator methionine of Orm1 by site-directed mutagenesis. pSH14HA-5S has 5 Ser to Ala changes (S29A, S32A, S34A, S35A, S36A). Primer sequences available upon request. pMK204-7-3HA is a *HIS3*-marked centromeric plasmid bearing HA-tagged *LAC1*, a gift from Scott Moye-Rowley (Kolaczowski et al., 2004). pJC104, a reporter for UPR, is a *URA3*-marked 2 μ plasmid bearing UPR-lacZ from Peter Walter's lab (Cox and Walter, 1996).

Mobility shift gels, Western blot, TAP pulldown, and enzyme assay

To detect differences in phosphorylation of Orm proteins, cell aliquots (0.9-1.4 OD₆₀₀) were resuspended in 180 μ l buffer (1.4 M sorbitol, 25 mM Tris, pH 8) and 45 μ l 85% trichloroacetic acid (TCA), and frozen in liquid nitrogen. Cell extracts were prepared by vortexing with glass beads 4 times 1 min. Extracts were collected after adding TCA (250 μ l 5%), and an additional 300 μ l 5% TCA was added to wash the beads. After 20 min on ice, precipitated protein was collected by centrifugation for 10 min in a microfuge. Pellets were washed with acetone, and after centrifugation for 2 min, pellets were dried for 10 min. Pellets were resuspended in 2% SDS, 10 mM Tris, pH 6.7 followed by sonication in a water bath. Protein concentration was determined by BCA assay (Pierce). Changes in phosphorylation of TAP-tagged Orm1 were detected by mobility differences detected after extended electrophoresis on 12% SDS polyacrylamide gels and Western blot with rabbit serum. HA-tagged Orm1 was analyzed on 10% SDS polyacrylamide gels without an extended run. Western blots were quantified using NIH Image.

For treatment with alkaline phosphatase, TCA-precipitated lysate pellets were solubilized in 1% SDS, 50 mM Tris, pH 8. Samples were adjusted to 0.2% SDS, 0.2% β -mercaptoethanol, 50 mM Tris, pH 8. Calf intestinal alkaline phosphatase (1.5 μ l/100 μ l protein) was added for 1h at 37°C.

For pull-downs, cells (10 OD₆₀₀) were lysed by vortexing with glass beads in the presence of buffer (1% NP40, 150 mM NaCl, 10 mM Tris pH 7.4). Lysates were centrifuged at 400 x *g* for 5 min to remove unbroken cells. TAP pull-down with IgG Sepharose (GE Healthcare) was normalized to protein content (300 μ g). IgG Sepharose was preincubated for 2h with

protein lysate without TAP protein (100 µg) to inhibit nonspecific binding. Pull-downs were normalized to lysate protein, and analyzed by SDS-PAGE and Western blot with anti-HA monoclonal antibody followed by reblot with rabbit serum.

For quantitating UPR, cells bearing UPR-lacZ were lysed by vortexing with glass beads in breaking buffer (20% glycerol, 1 mM DTT, 100 mM Tris, pH 8) with a protease inhibitor cocktail and phenylmethylsulfonyl fluoride, as described previously (Chang and Slayman, 1991). β galactosidase activity was measured as described previously (Rose et al., 1990), and activities were normalized to lysate protein measured by Bradford assay (Bradford, 1976).

Long chain base and ceramide measurements

For mass spectrometry, lipids were extracted with CHCl₃:MeOH (17:1; per vol) from 10 OD of cells containing 125 pmol C17 dihydrosphingosine and 170 pmol of C18-ceramide as internal standards (Ejsing et al., 2009). Long chain bases were analyzed in the positive ion mode 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 gas collision at a pressure of 8 mbar. [M+H]⁺ ions of phytosphingosine and [M-H]⁻ ions of ceramide species were quantified relative to the internal standards.

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Figure Legends

Fig. 1. *Orm1* phosphorylation is dependent on the TOR signaling pathway.

A. Phosphorylation of Orm1 is dependent on Sit4 and increased by rapamycin.

Exponentially growing cells [wild-type (SHY53) and *sit4Δ* (ACX198-1C)] were treated with myriocin (0.1 μg/ml) for 1h or rapamycin (200 nM) for 20 min at 30°C, and frozen in liquid nitrogen in the presence of TCA. Lysates were analyzed by Western blot after extended electrophoresis to resolve differences in extent of phosphorylation. Arrowheads indicate increased phosphorylation in wild-type cells after addition of myriocin and rapamycin.

B. Orm2 phosphorylation is not affected by rapamycin and independent of Sit4.

Exponentially growing wild-type cells with TAP-tagged Orm2 (ACX184-2B) and *sit4Δ* cells (194-1D) were incubated with myriocin (0.15 μg/ml) for 1h or rapamycin (200 nM) for 20 min at 30°C, and then frozen in liquid nitrogen in the presence of TCA.

C. Sphingolipid synthesis is decreased in *sit4Δ* cells. Cells were grown in SC medium at 30°C prior to lipid extraction and measurement of ceramide C and C', DHS, and PHS by mass spectrometry. Measurements were made in duplicate on two independent colonies.

Fig. 2. *Impaired Orm1 phosphorylation and concomitantly reduced ceramide in temperature-sensitive tap42-11 cells.*

(A & B) Phosphorylation of Orm1. Exponentially growing *TAP42⁺* and *tap42-11* cells were grown at 30°C in SC medium. Cells were then kept at 30°C or shifted to 37°C; after 10 min, myriocin (0.15 μg/ml) or rapamycin (200 nM) were added for 1h or 30 min, respectively. Cells were then frozen in liquid nitrogen in the presence of TCA. Lysate was analyzed by Western blot. (A) Western blot of TAP-tagged Orm1 (ACX216). (B) Left panel, Western blot of HA-tagged Orm1 (pSH14HA) in *TAP42⁺* cells (CY1077) and *tap42-11* (CY1078). Right panel, quantitation of Western blot. Maximally phosphorylated HA-Orm1 (topmost

band, arrow) is expressed as a percentage of total HA-Orm1 signal. HA-Orm1 in *tap42-11* cells (black bars), and *TAP42* cells (grey bars).

(C) Ceramide levels in *tap42-11* cells was determined by mass spectrometry. Cells were grown at 24°C in SC medium, and incubated at the respective temperature for 2 h prior to lipid extraction and measurement of ceramide C. Measurements were made in duplicate on two independent colonies.

(D) Western blot of HA-Orm1 (pSH14HA) in wild-type (HXX1-7C) and *npr1Δ* cells. Cells were incubated in SC-histidine medium in the presence and absence of myriocin (0.15 μg/ml) for 1h or rapamycin (200 nM) for 20 min at 30°C. Arrow shows impaired phosphorylation in response to rapamycin treatment.

Fig. 3. *Phosphorylation of Orm1 in response to Orm2/SPT status*

(A) Top panel, Western blot to analyze phosphorylation of HA-Orm1 (pSH14HA). Cells were treated +/- myriocin or rapamycin for 1h and 20 min, respectively, at 30°C. HA-Orm1 phosphorylation was compared in *orm1Δ* cells (HXX1-7B) with a chromosomal copy of *ORM2* (+), *orm1Δ orm2Δ* cells (HXX1-7D) (Δ), and *orm1Δ* cells (HXX1-7B) with a 2 micron plasmid overexpressing *ORM2* (pSH17) (+++). Asterisk indicates maximal HA-Orm1 phosphorylation in cells with high copy *ORM2*. As indicated, lysate was treated +/- alkaline phosphatase for 1h at 37°C. Arrow indicates mobility of dephosphorylated HA-Orm1. Bottom panel, growth of wild-type cells (HXX1-7C) bearing vector or *ORM2* 2μ (pSH17). Serial dilutions of cells were spotted on plates with SC-leucine medium and 560 ng/ml myriocin.

(B) Western blot to analyze constitutive phosphorylation of HA-Orm1 (pSH14HA) in *orm1Δ* (HXX1-7B) (lanes 1, 4), wild-type (HXX1-7C) (lanes 2, 5, 7), and *sac1Δ* cells (lanes 3, 6 8). Cells were stimulated as in (A). (C) Phosphorylation of 5S-A HA-Orm1 mutant in response to myriocin and rapamycin as in (A). Arrow indicates stimulated phosphorylation of HA-Orm1.

Fig. 4. Association of *Orm1* and *Orm2* with ceramide synthase

(A) *ORM2*⁺ (ACX191-2A), *orm2Δ* (225-1A), *lag1Δ* (ACX217-1A) cells with TAP-tagged chromosomal *ORM1* and a centromeric plasmid bearing *HA-LAC1* were incubated +/- myriocin (0.15 μg/ml) for 1h at 30°C in SC-histidine medium. Pull-down of HA-Lac1 by Orm1-TAP was also examined in cells (ACX191-2A) with high copy *ORM2* (pSH17). Control cells were transformed with *HA-LAC1* but without TAP-tagged *ORM1* (HXX1-7C). Lysate was prepared by vortexing cells with glass beads in the presence of 1% NP40. Input (lysate) is 10% of protein content used for pull-downs. Pull-downs, by incubation of lysate overnight with IgG Sepharose, were analyzed by SDS-PAGE and Western blot with anti-HA monoclonal antibody (middle panel). Orm1-TAP pull-down was confirmed by reblotting with rabbit serum (bottom panel).

(B) Pull-down using lysate from cells with TAP-tagged chromosomal *ORM2* (CHY 48) bearing a centromeric plasmid with *HA-LAC1*. Protocol as described in (A).

Fig. 5. Sphingolipid synthesis and ER stress response

(A) Ceramide and long chain base levels are decreased upon *ORM1* and *ORM2* overexpression. Wild-type (HXX1-7C) cells bearing 2μ plasmids with *ORM1* (pSH16) or *ORM2* (pSH17) were grown in SC medium at 30°C prior to lipid extraction and measurement of ceramide C and C', DHS, and PHS by mass spectrometry. Measurements were made in duplicate on two independent colonies.

(B) Orm1 phosphorylation is increased upon UPR induction. *orm1Δ* (HXX1-7B) or *orm1Δ orm2Δ* cells (HXX1-7D) bearing HA-tagged Orm1 (pSH14HA) were treated +/- myriocin (0.15 μg/ml), tunicamycin (1 μg/ml), or DTT (1 mM) for 1h. Cells were frozen in TCA before lysis by vortexing with glass beads. Lysates were normalized to protein content and analyzed by Western blot with anti-HA antibody. Arrow indicates absence of phosphorylation in response to tunicamycin and DTT in *orm2Δ* cells.

Fig. 6. *Constitutive UPR in orm1Δ orm2Δ cells*

Cells carrying a UPRE-lacZ reporter were grown at 30°C in synthetic complete medium minus uracil. After incubation +/- tunicamycin (1 µg/ml) for 1h, cell lysate was prepared and β galactosidase activity was measured. Measurements were made in duplicate on at least 2 independent colonies. Constitutive UPR in the absence of the Orm proteins is Ire1-dependent and also dependent on Mpk1/Slt2 and Mid2.

Fig. 1

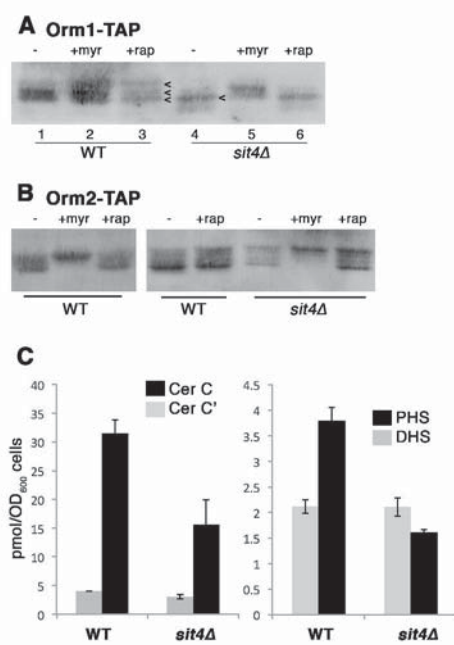


Fig. 2

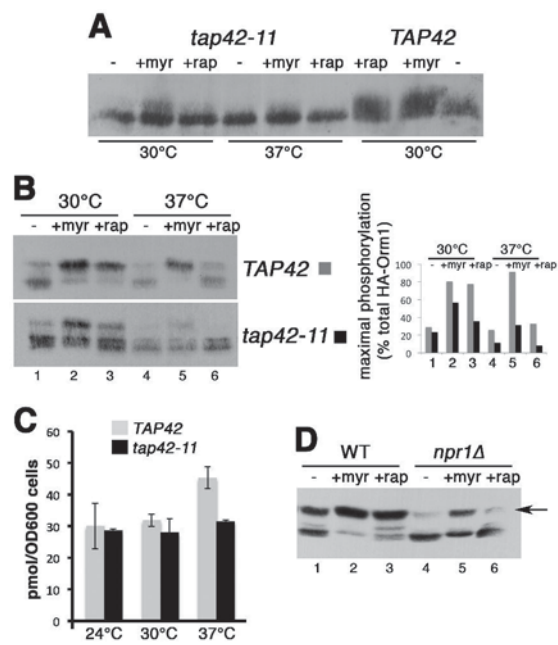


Fig. 4

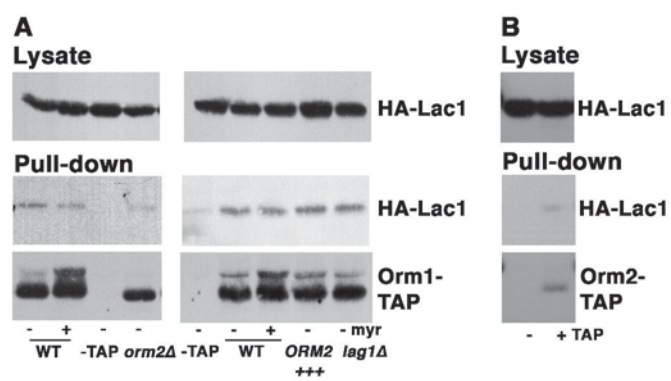
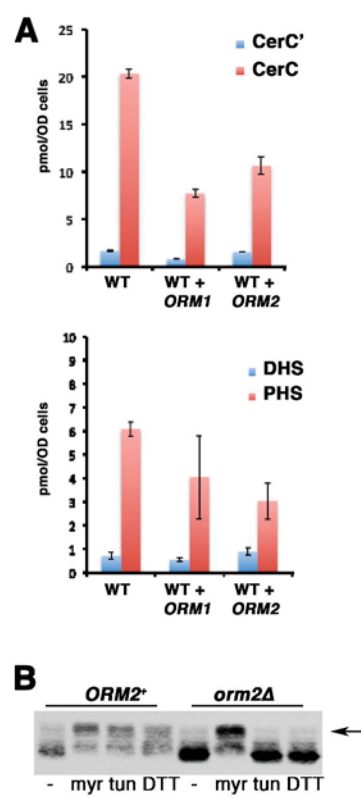


Fig. 5



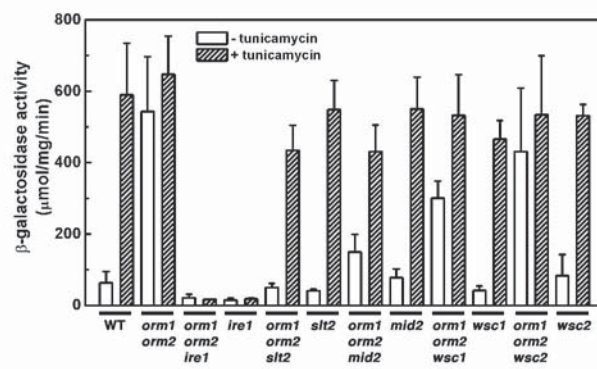


Fig. 6