Regulation of sphingolipid synthesis through Orm1 and Orm2 in yeast

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Summary

Sphingolipids are crucial components of membranes, and sphingolipid metabolites serve as signaling molecules. Yeast Orm1 and Orm2 belong to a conserved family of ER membrane proteins that regulate serine palmitoyltransferase, which catalyzes the first and rate-limiting step in sphingolipid synthesis. We now show that sphingolipid synthesis through 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 for regulating sphingolipid synthesis is through control of Orm2 protein level. The 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 that 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 might signal to the ER from the plasma membrane because UPR activation is dependent on a cell surface sensor and the mitogen-activated protein kinase (MAPK) cell wall integrity pathway. Thus, sphingolipid synthesis and the UPR are coordinately regulated.

Key words: ER stress response, Endoplasmic reticulum, Protein phosphorylation, Sphingolipid synthesis

Introduction

Membrane biogenesis during cell growth and proliferation involves coordination of protein and lipid synthesis. In response to nutritional conditions, protein synthesis is modulated by multiple signaling pathways (Smets et al., 2010). One of these is target of rapamycin (TOR), 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 crucial structural components of membranes and contribute 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 production of long chain bases 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 a 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 adjusts SPT activity to maintain sphingolipid homeostasis (Breslow et al., 2010). In cells depleted of Orm1 and Orm2 (*orm1Δ orm2Δ* cells), dysregulation of sphingolipid synthesis results in pleiotropic phenotypes, including impaired growth and constitutive activation of the 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 through Orm1. In this study, we show that Orm1 phosphorylation and sphingolipid synthesis are dependent on the Sit4–Tap42 complex, a downstream target of the rapamycin-sensitive TOR protein kinase complex, TORC1. Another mechanism for regulating sphingolipid synthesis is by changing the amount of Orm2 protein. 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 tandem affinity

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purification (TAP)-tagged Orm1 was detected as electrophoretic mobility shifts (Fig. 1). Orm1 is constitutively phosphorylated on multiple residues (Breslow et al., 2010) (Fig. 3). 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 were treated with myriocin to inhibit SPT, phosphorylation of Orm1–TAP was increased and 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 treatment for 20 minutes (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).

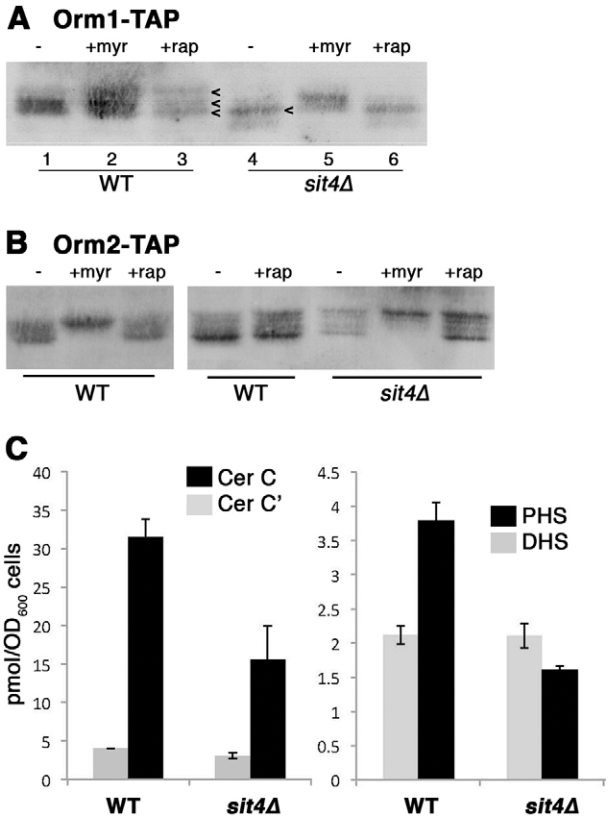

Fig. 1. Orm1 phosphorylation is dependent on the TOR signaling pathway. (**A**) Phosphorylation of Orm1 is dependent on Sit4 and increased by rapamycin. Exponentially growing cells [wild-type (SHY53) and *sit4Δ* (ACX198-1C)] were treated with myriocin (0.15 μg/ml) for 1 hour or rapamycin (200 nM) for 20 minutes at 30℃, and frozen in liquid nitrogen in the presence of TCA. Lysates were analyzed by western blot after extended electrophoresis to resolve differences in the extent of phosphorylation. Arrowheads indicate increased phosphorylation in wild-type cells after addition of myriocin and rapamcin. (**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 1 hour or rapamycin (200 nM) for 20 minutes at 30℃, and then frozen in liquid nitrogen in the presence of TCA. (**C**) Sphingolipid synthesis is decreased in *sit4Δ* cells. Cells were grown in synthetic complete (SC) medium at 30℃ prior to lipid extraction and measurement of ceramide C (t18:0/C26:0) and C′ (t18:0/C24:0), dihydrosphingosine (DHS) and phytosphingosine (PHS) by mass spectrometry. Measurements were made in duplicate on two independent colonies.

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, the electrophoretic mobility of Orm1–TAP and Orm2–TAP was assessed in *sit4Δ* cells. As shown in Fig. 1A, basal phosphorylation of Orm1 appeared decreased in *sit4Δ* cells, and response to myriocin and rapamycin was abrogated. By contrast, basal phosphorylation of Orm2 and response to myriocin were unaffected in *sit4Δ* cells.

In *sit4Δ* cells, decreased Orm1 phosphorylation correlated with decreased long chain base and ceramide levels, as 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℃ and lethality at 37℃ (Cherkasova and Hinnebusch, 2003). *TAP42*$^{+}$ and *tap42-11* cells were grown at 30℃ and shifted to 37℃ in the presence or absence of myriocin for 1 hour. A comparison of Orm1–TAP electrophoretic mobility is shown in Fig. 2A. Stimulated phosphorylation in response to rapamycin was barely detectable in *tap42-11* cells at 30℃ and undetectable at 37℃.

To improve resolution of electrophoretic mobility shifts, Orm1 protein was tagged with a single hemagglutinin (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). 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 quantified by scanning western blots and plotting maximally phosphorylated HA–Orm1 (top band) as a percentage of total HA–Orm1 (Fig. 2B, right). In comparison with wild-type cells, rapamycin-induced phosphorylation in *tap42-11* cells was reduced at 30℃ (Fig. 2B, lane 3). At 37℃, response to rapamycin in *tap42-11* cells was undetectable (Fig. 2B, compare lanes 3 and 6). At 37℃, basal and myriocin-stimulated phosphorylation of Orm1 were significantly reduced in *tap42-11* cells (Fig. 2B, lanes 4 and 5).

Ceramide levels were compared in wild-type and *tap42-11* cells by mass spectrometry. Cells were grown at 24℃ and then shifted to either 30℃ or 37℃ for 2 hours before lipid analysis. In *tap42-11* cells, a shift to 37℃ resulted in decreased ceramide levels (Fig. 2C). Upon Tap42 inactivation, the temporal correspondence between diminished levels of ceramide, and reduced Orm1 phosphorylation (Fig. 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 was abrogated in response to rapamycin (Fig. 2D, compare lanes 3 and 6, arrow) and Orm1

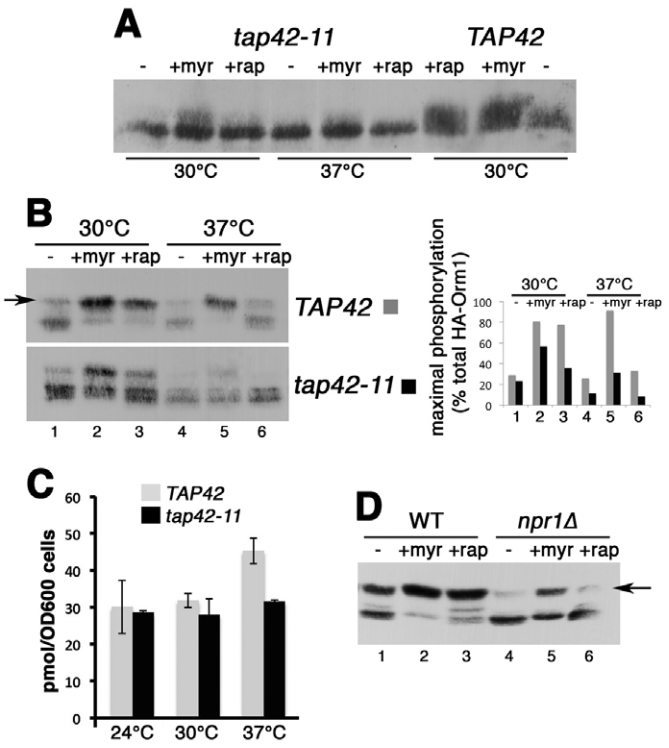

Fig. 2. Impaired Orm1 phosphorylation and concomitantly reduced ceramide in temperature-sensitive *tap42-11* cells. (**A**,**B**) Phosphorylation of Orm1. Exponentially growing *TAP42*$^{+}$ and *tap42-11* cells were grown at 30℃ in synthetic complete (SC) or SC-histidine medium. Cells were then kept at 30℃ or shifted to 37℃. After 10 minutes, myriocin (0.15 μg/ml) or rapamycin (200 nM) were added and the cells incubated for 1 hour or 30 minutes, 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: Western blot of HA-tagged Orm1 (pSH14HA) in *TAP42*$^{+}$ cells (CY1077) and *tap42-11* (CY1078). Right: Quantification of western blot. Maximally phosphorylated HA–Orm1 (top 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* and *tap42-11* cells were determined by mass spectrometry. Cells were grown at 24℃ in SC medium and incubated at the indicated temperatures for 2 hours prior to lipid extraction and measurement of ceramide C (t18:0/C26:0). 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 1 hour or rapamycin (200 nM) for 20 minutes at 30℃. Arrow shows impaired phosphorylation in response to rapamycin treatment.

phosphorylation in response to myriocin was impaired (Fig. 2D, compare lanes 2 and 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 was heterogeneously phosphorylated with lesser phosphorylated forms predominating (Fig. 3A, lane 7). In *orm2Δ* cells, HA–Orm1 was dephosphorylated with an electrophoretic mobility similar to that from *ORM2*$^{+}$ cells treated with alkaline phosphatase (Fig. 3A, compare lanes 8 and 9). HA–Orm1 in *orm2Δ* cells appears fully dephosphorylated because no effect on its mobility was detected after treatment with alkaline phosphatase (Fig. 3A, compare lanes 11 and 12, arrow). However, in *orm2Δ* cells, HA–Orm1 phosphorylation was increased in response to myriocin addition (Fig. 3A, lane 3), suggesting that SPT activity is a major influence on Orm1 phosphorylation state. When Orm2 was overexpressed, HA–Orm1 phosphorylation was constitutively increased (Fig. 3A, lane 4, asterisk). Even so, in cells with high copy *ORM2*, growth on plates with low dose myriocin was compromised (Fig. 3A, bottom panels), 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 SPT, Orm1, Orm2, Tsc3 and Sac1) (Breslow et al., 2010). Fig. 3B, lane 3, shows that Orm1 phosphorylation was constitutively increased in *sac1Δ* cells. Because there is a chromosomal copy of *ORM1* in *sac1Δ* cells in addition to HA–Orm1, two controls are shown. Basal phosphorylation of HA–Orm1 was increased in cells with chromosomal *ORM1* (Fig. 3B, lane 2) in comparison with cells in which HA–Orm1 was the sole copy of *ORM1* (Fig. 3B, 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). Similarly to the response to myriocin, increased phosphorylation of Orm1 in *sac1Δ* cells might reflect a compensatory 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 N-terminus (Breslow et al., 2010). Serines 32 and 34 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 (5S-A Orm1) that has five serine-to-alanine changes (S29A, S32A, S34A, S35A, S36A). Constitutive phosphorylation of mutant Orm1 was reduced compared to that of wild-type Orm1 (Fig. 3C, lanes 5 and 8). Stimulated phosphorylation of Orm1 in response to rapamycin was abrogated (Fig. 3C, lane 11). Response to myriocin was impaired as mobility of mutant Orm1 shifted up slightly (compare lane 2 with lane 3) but the maximally phosphorylated band was not present (Fig. 3C, 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 the possibility that ceramide synthase activity is regulated by the Orm proteins independently of SPT. Ceramide synthase comprises three ER membrane proteins, Lac1, Lag1 and Lip1 (Vallée and Riezman, 2005). To detect physical association with Orm1–TAP, an HA-tagged Lac1 construct was used in pull-down experiments. Fig. 4A shows the presence of

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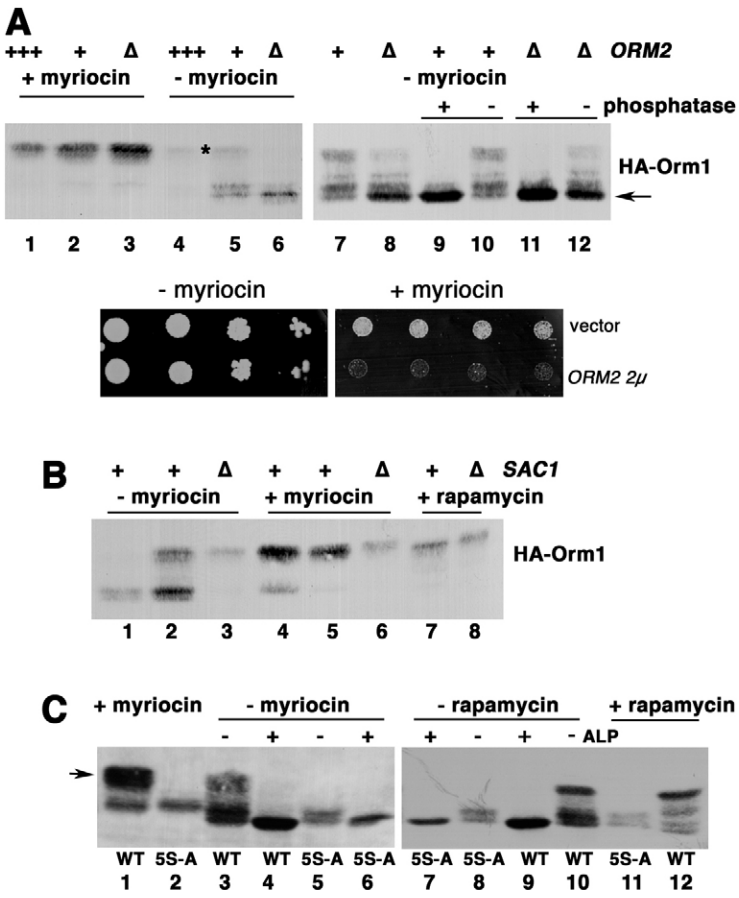

Fig. 3. Phosphorylation of Orm1 in response to Orm2/SPT status. (**A**) Top: Western blot to analyze phosphorylation of HA–Orm1 (pSH14HA). Cells were treated with or without myriocin for 1 hour or rapamycin for 20 minutes at 30℃. HA–Orm1 phosphorylation was compared in *orm1Δ* cells (HXX1-7B) with a chromosomal copy of *ORM2* (+), in *orm1Δ orm2Δ* cells (HXX1-7D) (*Δ*) and in *orm1Δ* cells (HXX1-7B) with a 2 μ plasmid overexpressing *ORM2* (pSH17) (+++). Asterisk indicates maximal HA–Orm1 phosphorylation in cells with high copy *ORM2*. As indicated, lysate was treated with or without alkaline phosphatase for 1 hour at 37℃. Arrow indicates mobility of dephosphorylated HA–Orm1. Bottom: Growth of wild-type cells (HXX1-7C) bearing vector or *ORM2 2* μ (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. Arrowhead indicates stimulated phosphorylation of HA–Orm1.

HA-tagged Lac1 in an Orm1–TAP pull-down under non-denaturing conditions. HA–Lac1 was also associated with Orm2–TAP (Fig. 4B). Association of Orm1 with Lac1 was independent of Lag1 (Fig. 4A, right). Orm1 association with HA–Lac1 also appeared to be independent of Orm1 phosphorylation state because the association remained intact after addition of myriocin or upon *ORM2* overexpression when Orm1 phosphorylation is increased. Orm1–TAP interaction with HA–Lac1 was unaffected in *orm2Δ* cells when Orm1 is dephosphorylated (Fig. 4A, left). 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 the Orm2 protein level could be 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 resulted in decreased levels of long chain base and ceramide; Orm1 overexpression also induced decreased ceramide levels. These findings predict that sphingolipid synthesis is decreased by Orm2 protein induced during the ER stress response.

To examine the response of Orm1 to ER stress, cells were treated with tunicamyin, an inhibitor of N-linked glycosylation, or with the reducing agent DTT. As shown in Fig. 5B, Orm1 phosphorylation was stimulated by tunicamycin and DTT treatments. Strikingly, in the absence of Orm2, Orm1 phosphorylation did not respond to tunicamycin or DTT (Fig. 5B, arrow). In the absence of Orm2, Orm1 phosphorylation still increased 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 mitogen-activated protein kinase (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 because 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 *UPRE-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 was also dependent on Ire1 (Fig. 6). Remarkably, signaling of constitutive UPR in *orm1Δ orm2Δ* cells also required the MAPK Slt2/Mpk1 (Fig. 6). Activation of UPR in response to tunicamycin addition was not impaired in *slt2Δ orm1Δ orm2Δ* cells.

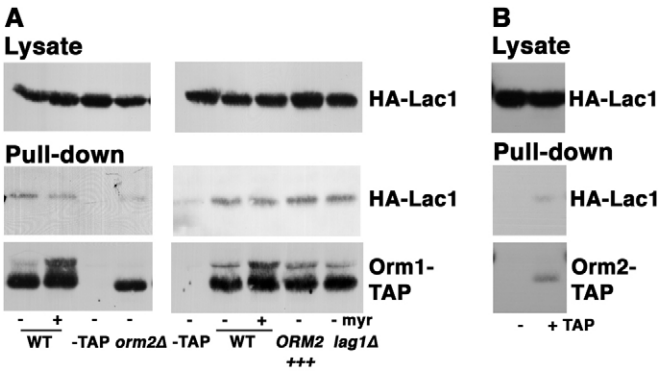

Fig. 4. Association of Orm1 and Orm2 with ceramide synthase. (**A**) *ORM2*$^{+}$ (ACX191-2A), *orm2Δ* (225-1A) and *lag1Δ* (ACX217-1A) cells with TAP-tagged chromosomal *ORM1* and a centromeric plasmid bearing *HA-LAC1* were incubated with or without myriocin (0.15 μg/ml) for 1 hour at 30℃ in SC-histidine 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) was 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 was as described for A.

The MAPK signaling cascade plays a well-established role in maintaining cell wall integrity through 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, *UPRE-lacZ* activity was assayed in *mid2Δ orm1Δ orm2Δ* cells. Fig. 6 shows that signaling of constitutive UPR in *orm1Δ orm2Δ* cells was prevented in *mid2Δ* cells. Dependence on Mid2 suggests that signaling occurs from the plasma membrane to initiate the UPR response at the ER. In contrast to loss of Mid2, constitutive UPR in *orm1Δ orm2Δ* cells appeared to be less or insignificantly affected by loss of the cell wall integrity sensors Wsc1 and Wsc2 (Fig. 6).

Discussion

A major finding of this study is identification of a signaling pathway that regulates sphingolipid synthesis through Orm1 protein. 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℃, a decrease in ceramide levels accompanies impaired Orm1 phosphorylation (Fig. 2C). These results show that sphingolipid synthesis is regulated through Orm1 phosphorylation by the TORC1 signaling pathway, which coordinates membrane biogenesis with cell growth in response to environmental conditions.

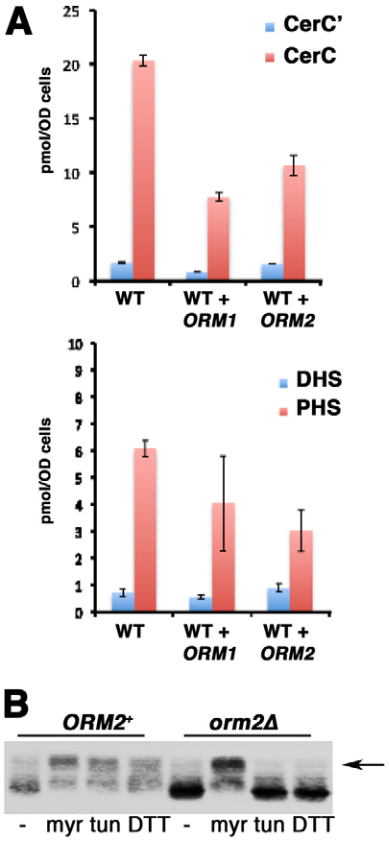

Fig. 5. Sphingolipid synthesis and ER stress response. (**A**) Ceramide and long chain base levels are decreased upon *ORM1* and *ORM2* overexpression. Wild-type (HXX1-7C) cells bearing 2μ plasmids with *ORM1* (pSH16) or *ORM2* (pSH17) were grown in SC medium at 30℃ prior to lipid extraction and measurement of ceramide C (t18:0/C26:0) and C′ (t18:0/C24:0), dihydrosphingosine (DHS) and phytosphingosine (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 with or without myriocin (myr; 0.15 μg/ml), tunicamycin (tun; 1 μg/ml) or DTT (1 mM) for 1 hour. 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.

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 through Npr1. Orm1 residues targeted by the TOR signaling pathway include Ser29, Ser32 and Ser34–36 because the 5S-A Orm1 mutant fails to respond to rapamycin (Fig. 3C). Because Orm1 phosphorylation in response to myriocin is only reduced in *npr1Δ* cells, Orm1 is probably phosphorylated at additional residues by one or more signaling pathway(s) that have not yet been identified. Previous work suggested that Orm1 residues Ser51–53 are phosphorylated in response to myriocin (Breslow et al., 2010)]. Similarly, the signaling pathways regulating Orm2 are not yet known, but appear to be independent of TORC1 because Orm2 phosphorylation is rapamycin-independent (Fig. 1B).

The Orm proteins respond to the status of the sphingolipid synthesis pathway: increased phosphorylation of both Orm

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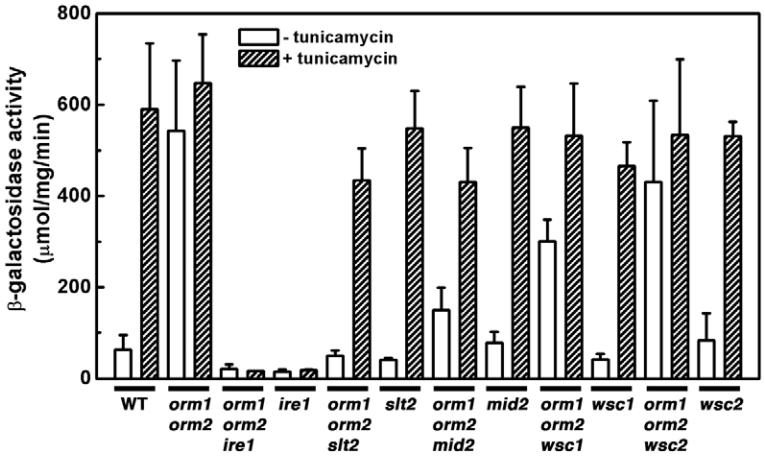

Fig. 6. Constitutive UPR in *orm1Δ orm2Δ* cells. Cells carrying a *UPRE-lacZ* reporter were grown at 30℃ in synthetic complete medium minus uracil. After incubation with or without tunicamycin (1 μg/ml) for 1 hour, cell lysate was prepared and β-galactosidase activity measured. Measurements were made in duplicate on at least two independent colonies. Constitutive UPR in the absence of the Orm proteins is Ire1-dependent and also dependent on Mpk1/Slt2 and Mid2.

proteins relieves repression of SPT (and restores homeostatic SPT activity when myriocin is added) (Breslow et al., 2010). Although both Orm proteins respond to SPT activity, Orm1 also responds to Orm2 status, and Orm1 phosphorylation changes as a compensatory response to increased Orm2 or loss of Orm2 (Fig. 3): Orm1 phosphorylation increases (to derepress SPT activity) when Orm2 is overexpressed, and Orm1 is dephosphorylated in the absence of Orm2 (to increase SPT repression). These results have important physiological implications because the Orm2 protein level is adjusted by ER stress conditions (Han et al., 2010). Genetic evidence indicates overlapping functions for Orm1 and Orm2; nevertheless, because *orm2Δ* cells have a subset of phenotypes associated with *orm1Δ orm2Δ* double mutants whereas *orm1Δ* cells have no observable phenotype (Han et al., 2010), it appears that Orm2 has a prevailing influence on SPT activity.

In the absence of the Orm proteins, SPT activity is derepressed and long chain base accumulates (Breslow et al., 2010; Han et al., 2010). Ceramide levels are decreased in *orm1Δorm2Δ* cells (Han et al., 2010). Impaired de novo ceramide synthesis in *orm1Δ orm2Δ* cells is supported by accumulation of very long fatty acid levels [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 Orm1 and Orm2 with the ceramide synthase subunit, Lac1 (Fig. 4). Lac1 was not present in the SPOTS protein complex identified by Orm pull-down (Breslow et al., 2010). Detergent conditions might possibly affect detection of Orm association with Lac1. Physical association between Lac1 and Orm1 remains unaffected by myriocin treatment or *ORM2* overexpression (when Orm1 phosphorylation is increased) or in the absence of Orm2 (when Orm1 is dephosphorylated). Because Lac1 and Lag1 are functionally redundant (Schorling et al., 2001), Orm1–Lac1 association in the absence of Lag1 indicates that interaction occurs with a catalytically active subunit. Our results suggest a more direct role for the Orms in regulating ceramide synthesis, although further work is necessary to determine the mechanism by which the Orms can affect ceramide synthase activity.

In the absence of the Orm proteins, UPR is constitutively activated (Fig. 6) (Han et al., 2010). Other reports have linked ER stress and UPR activation with the 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 *orm1Δ orm2Δ* 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, i.e. a role in 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 might 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 MAPK 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 through ER–plasma membrane junctions (Stefan et al., 2011). 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 because 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). 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 levels (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 reflect the major factor by which sphingolipid synthesis is repressed by ER stress. It has been proposed that increased phospholipid

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synthesis and membrane expansion are responses that alleviate ER stress (Schuck et al., 2009). Accordingly, we suggest that during the ER stress response, repressed sphingolipid synthesis changes 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 the ER stress response is triggered by lipid imbalance and that chronic ER stress plays a crucial detrimental role in lipotoxicity (Brookheart et al., 2009). The best evidence that sphingolipid dysregulation contributes to metabolic disease comes from studies showing that inhibition of sphingolipid synthesis ameliorates disease in animal models (Summers, 2010). In this regard, work in yeast should continue to provide insight into the molecular mechanisms regulating sphingolipid homeostasis and the response to lipid dysregulation.

Materials and Methods

Strains and plasmids

Standard yeast media and genetic manipulations were as described previously (Sherman et al., 1986). 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 a cross between *sit4Δ* and ACX191-2A. CY1077 and CY1078 are *MAT***a** *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 a 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 five serine-to-alanine 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, University of Iowa, IA (Kolaczkowski et al., 2004). pJC104, a reporter for UPR, is a *URA3*-marked 2μ plasmid bearing *UPRE-lacZ* from the Peter Walter laboratory, University of California at San Francisco, CA (Cox and Walter, 1996).

Mobility shift gels, western blot, TAP pull-down and enzyme assay

To detect differences in phosphorylation of Orm proteins, cell aliquots (0.9–1.4 OD_{600}) were resuspended in 180 μl buffer (1.4 M sorbitol, 25 mM Tris, pH 8) and 45 μl 85% trichloroacetic acid (TCA), and frozen in liquid nitrogen. Cell extracts were prepared by vortexing with glass beads four times for 1 minute. Extracts were collected after adding 5% TCA (250 μl), and an additional 300 μl of 5% TCA was added to wash the beads. After 20 minutes on ice, precipitated protein was collected by centrifugation for 10 minutes in a microfuge. Pellets were washed with acetone, centrifuged for 2 minutes and dried for 10 minutes. 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 software.

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 μl/100 μl protein) was added and samples incubated for 1 hour at 37°C.

For pull-down assays, cells (10 OD_{600}) 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 ***g*** for 5 minutes to remove unbroken cells. TAP pull-down with IgG Sepharose (GE Healthcare) was normalized to protein content (300 μg). IgG Sepharose was preincubated for 2 hours 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 quantification of UPR, cells bearing *UPRE-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 as measured by the Bradford assay (Bradford, 1976).

Measurement of long chain base and ceramide

For mass spectrometry, lipids were extracted with $CHCl_3$ and MeOH (17:1 by volume) 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 ceramide in the negative ion mode on a Bruker Esquire HCT ion trap mass spectrometer (electrospray ionization) at a flow rate of 180 μl/hour 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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Note added in proof

While the manuscript was in review, two papers reported that Orm protein phosphorylation is dependent on TORC2-dependent Ypk1 protein kinase (Roelants et al., 2011; Sun et al., 2012).

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