Orm protein phosphoregulation mediates transient sphingolipid biosynthesis response to heat stress via the Pkh-Ypk and Cdc55-PP2A pathways

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ABSTRACT **Sphingoid intermediates accumulate in response to a variety of stresses, including heat, and trigger cellular responses. However, the mechanism by which stress affects sphingolipid biosynthesis has yet to be identified. Recent studies in yeast suggest that sphingolipid biosynthesis is regulated through phosphorylation of the Orm proteins, which in humans are potential risk factors for childhood asthma. Here we demonstrate that Orm phosphorylation status is highly responsive to sphingoid bases. We also demonstrate, by monitoring temporal changes in Orm phosphorylation and sphingoid base production in cells inhibited for yeast protein kinase 1 (Ypk1) activity, that Ypk1 transmits heat stress signals to the sphingolipid biosynthesis pathway via Orm phosphorylation. Our data indicate that heat-induced sphingolipid biosynthesis in turn triggers Orm protein dephosphorylation, making the induction transient. We identified Cdc55–protein phosphatase 2A (PP2A) as a key phosphatase that counteracts Ypk1 activity in Orm-mediated sphingolipid biosynthesis regulation. In total, our study reveals a mechanism through which the conserved Pkh-Ypk kinase cascade and Cdc55-PP2A facilitate rapid, transient sphingolipid production in response to heat stress through Orm protein phosphoregulation. We propose that this mechanism serves as the basis for how Orm phosphoregulation controls sphingolipid biosynthesis in response to stress in a kinetically coupled manner.**

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INTRODUCTION

Sphingoid intermediates, including sphingoid bases, sphingoid base phosphates, and ceramides (Figure 1), play important roles in regulation of cell growth, differentiation, senescence, and apoptosis (Hannun and Obeid, 2008; Dickson, 2010; Nikolova-Karakashian and Rozenova, 2010). Serine palmitoyltransferase (SPT) mediates the rate-limiting first step in sphingolipid biosynthesis (Figure 1). Despite the importance of sphingoid intermediates as bioactive molecules, the regulation of sphingolipid biosynthesis through SPT is not well understood (Cowart and Hannun, 2007). A recent study revealed that yeast Orm proteins, encoded by *ORM1* and *ORM2*, form a conserved complex with SPT and that their phosphorylation status affects sphingolipid production (Breslow *et al.*, 2010; Figure 1). The authors proposed that sphingolipid levels feedback regulate Orm protein phosphorylation, thus mediating sphingolipid homeostasis (Breslow *et al.*, 2010). However, several important questions related to this model need answers. For example, whether and which sphingolipid species affect Orm phosphorylation are not known. In addition, how temporal regulation of Orm phosphorylation relates to dynamic changes in sphingolipid biosynthesis is not

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Abbreviations used: *Cg*, *Candida glabrata*; DHS, dihydrosphingosine; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; PHS, phytosphingosine; PP2A, phosphatase 2A; Sph, sphingosine; SPT, serine palmitoyltransferase; TCA, trichloroacetic acid; Ypk1, yeast protein kinase 1.

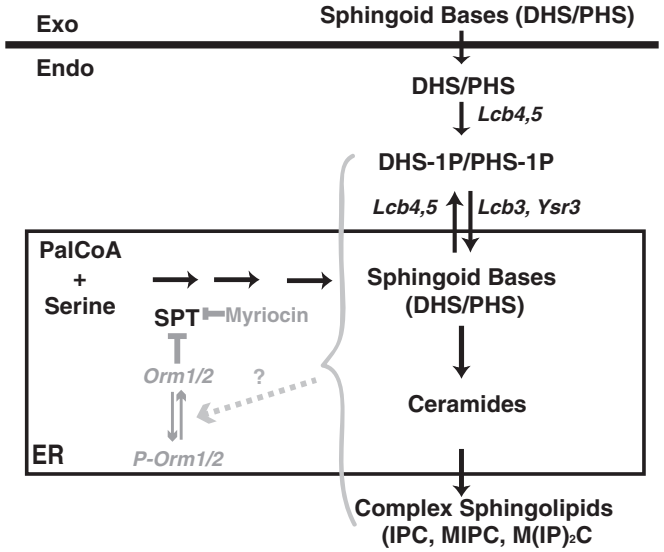

FIGURE 1: Schematic diagram of yeast sphingolipid biosynthesis from endogenous and exogenous precursors. Myriocin is a potent inhibitor of SPT (Sun *et al.*, 2000), which is the first and rate-limiting enzyme of the sphingolipid biosynthesis pathway (Buede *et al.*, 1991). Previous studies proposed that Orm proteins are dynamic negative regulators of SPT and their activities are inversely proportional to sphingolipid levels (Breslow *et al.*, 2010). Exogenous sphingoid bases (DHS and PHS) can be converted into ceramides in the endoplasmic reticulum after phosphorylation and dephosphorylation by the indicated enzymes and can then be incorporated into complex sphingolipids (Qie *et al.*, 1997; Nagiec *et al.*, 1998; Funato *et al.*, 2003).

known. Answers to these questions are required to better understand Orm-mediated sphingolipid homeostasis.

Another important question concerns how Orm-mediated sphingolipid homeostasis may function in a physiological context. Increasing evidence suggests that various stimuli trigger accumulation of sphingoid intermediates, which in turn function as bioactive molecules mediating cellular responses (Hannun and Obeid, 2008; Dickson, 2010). For instance, heat stress–induced sphingoid intermediates act as signaling molecules to induce cellular responses such as translation initiation of heat shock proteins, gene regulation, and cell cycle arrest (Dickson *et al.*, 1997; Mao *et al.*, 1999; Jenkins and Hannun, 2001; Cowart and Hannun, 2005; Cowart *et al.*, 2010; Meier *et al.*, 2006; Han *et al.*, 2010). It is striking that several groups demonstrated that heat stress induces sphingolipid biosynthesis in a rapid and transient manner (Dickson *et al.*, 1997; Jenkins *et al.*, 1997; Jenkins, 2003; Wells *et al.*, 1998; Mao *et al.*, 1999; Skrzypek *et al.*, 1999), suggesting that biosynthesis of sphingoid intermediates in response to stresses requires precise temporal regulation. We hypothesize that the dynamic changes in sphingoid intermediate levels upon heat stress may be caused by and/or may lead to changes of Orm phosphorylation. If this is the case, heat stress could serve as a model system to address the mechanism of how Orm phosphoregulation functions in the response of sphingolipid biosynthesis to stress in general.

A recent study showed that yeast protein kinases 1 and 2 (Ypk1/2), the homologues of mammalian serum- and glucocorticoid-inducible kinase (Casamayor *et al.*, 1999), directly phosphorylate Orm proteins in vitro (Roelants *et al.*, 2011). This result suggests that Ypk kinase may regulate sphingolipid homeostasis through its phosphorylation of the Orm proteins. However, whether Ypk kinase activity indeed affects sphingolipid production has not been examined. Of interest, the Ypk kinases were previously considered to be downstream of heat-induced sphingolipid base accumulation (Sun *et al.*, 2000; Friant *et al.*, 2001; Liu *et al.*, 2005; Hannun and Obeid, 2008; Dickson, 2010). Thus, testing for a possible role for Ypk kinase activity in the sphingolipid biosynthesis response to heat stress is an important goal.

Of importance, the Orm phosphorylation state is set not only by protein kinases, but also by phosphatases. A reasonable prediction is that the phosphatases acting on the Orm proteins are likely to be involved in and/or regulated by sphingolipid levels. Protein phosphatase 2A (PP2A) was identified as an attractive candidate for a ceramide-activated protein phosphatase in yeast (Nickels and Broach, 1996). Inactivation of the regulatory subunits (such as Cdc55) or the catalytic subunits of PP2A was reported previously to suppress the endocytic defects of a mutant with impaired SPT activity (Friant *et al.*, 2000), suggesting that PP2A may be involved in sphingolipid production. Because PP2A was suggested previously to be involved in multiple cellular signaling pathways (Jiang, 2006), the challenge is to identify a specific relationship between Cdc55-PP2A and the Orm proteins for regulation of sphingolipid biosynthesis.

In this article, by addressing the questions just raised, we demonstrate how Orm phosphorylation regulation controls sphingolipid biosynthesis in response to heat stress in a kinetically coupled manner. We identify a signaling pathway by which the conserved Pkh-Ypk signaling cascade and Cdc55-PP2A facilitate rapid, transient sphingolipid production upon heat stress through precise regulation of Orm protein phosphorylation. Our study will therefore provide a foundation for future studies of sphingolipid-related responses to other stimuli.

RESULTS

Orm2 is the major Orm species in budding yeast

A 3XFLAG sequence was inserted at the 5′ end of the genes *ORM1* and *ORM2*, which encode the two homologous yeast Orm proteins. Because Orm2 expression was at least 10 times higher than Orm1 expression (Supplemental Figure S1a) and because 3XFLAG-Orm1 in an *orm2Δ* strain, but not 3XFLAG-Orm2 in an *orm1Δ* strain, caused slow cell growth (Supplemental Figure S1, b and c), we concluded that Orm2 is the major Orm protein. Consistently, *orm2Δ* cells but not *orm1Δ* cells showed perturbation in sphingolipid homeostasis (Han *et al.*, 2010). These results suggested that Orm2 provides the majority of the Orm protein function. Thus, we decided to monitor Orm2 phosphorylation in our studies. As shown in Figure 2a, four groups of Orm2 bands were observed when resolved by phosphate-affinity gel, which is widely used for the analysis of phosphoprotein isotypes (Kinoshita *et al.*, 2006). As previously reported (Breslow *et al.*, 2010), the slower-migrating bands in the top three groups were confirmed to be phosphorylated forms of Orm2 (Supplemental Figure S2). On the basis of the principle responsible for protein separation on phosphate-affinity gels (Kinoshita *et al.*, 2006), the bands in the top three groups are likely to be phosphorylated to different extents, with the slowest-migrating species being the most highly phosphorylated. The bands in the third group observed by Western blotting are often overexposed under the conditions that allow us to observe bands in all four groups (Figure 2a). Thus, we monitored changes in intensity of groups 1, 2, and 4 as clear indicators of changes in Orm2 phosphorylation status.

Exogenously provided sphingoid bases are sufficient to induce rapid Orm2 dephosphorylation

We examined how exogenously provided sphingoid bases, which are early sphingoid intermediates (Figure 1), affect Orm2 phosphorylation status. As shown in Figure 2, b and c, dephosphorylated Orm2, seen as the fastest-migrating bands (the fourth group), increased in

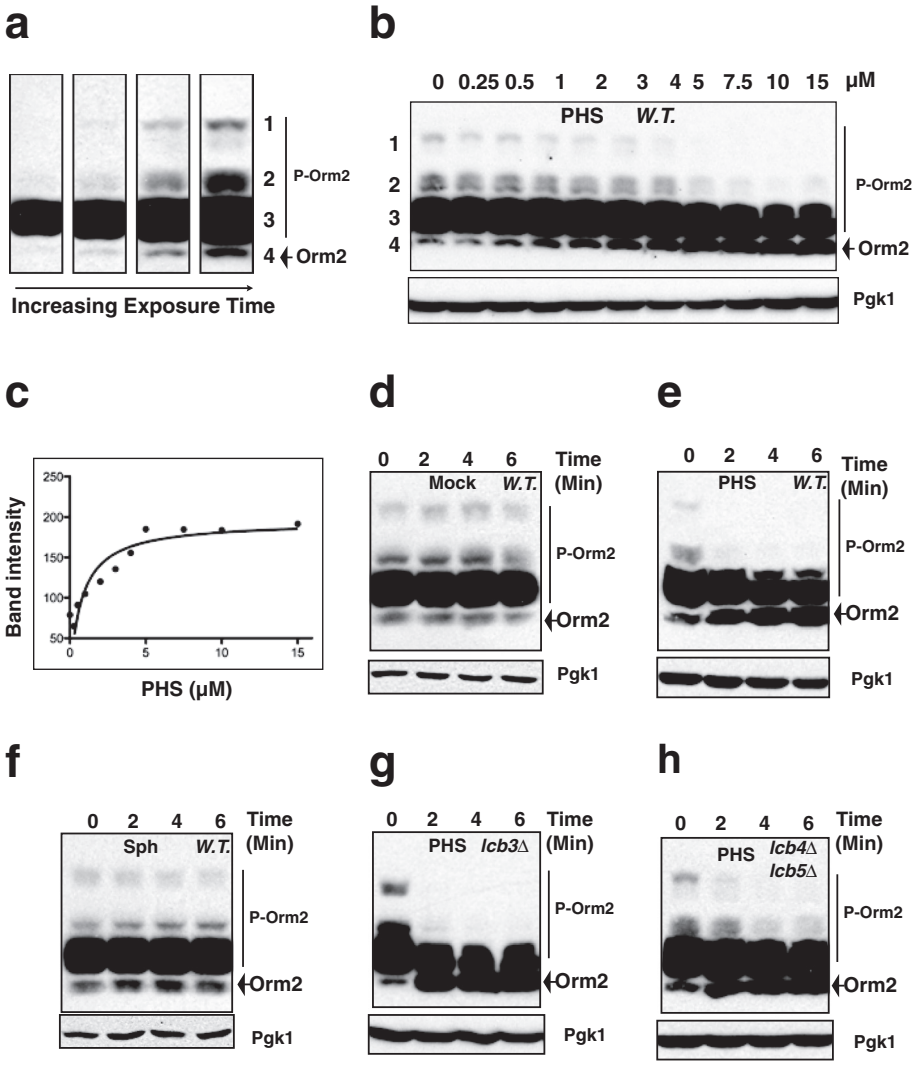

FIGURE 2: Exogenously provided sphingoid bases are sufficient to induce rapid Orm2 dephosphorylation. (a, b, d–h), Western blots showing Orm2 phosphorylation patterns after separation of the indicated yeast cell extracts on phosphate-affinity gels (top). P-Orm2 indicates phosphorylated forms of Orm2. We used 3-phosphoglycerate kinase 1 (Pgk1) as a loading control (bottom). All cells were grown to early log phase in YPD at 25°C before the indicated treatments. Cell extracts were prepared as described in *Materials and Methods*. (a) The majority of the Orm2 is moderately phosphorylated. Western blotting analysis of cell exacts from wild-type cells expressing 3XFLAG-Orm2 at its endogenous locus with different exposure times: 30 s and 1, 3, and 6 min (left to right). (b, c) Exogenous phytosphingosine (PHS) induces Orm2 dephosphorylation in a concentration-dependent manner. Wild-type cells expressing 3XFLAG-Orm2 at its endogenous locus were cultured in media containing the indicated concentrations of PHS (dissolved in methanol) for 10 min. The intensities of the fastest-migrating 3XFLAG Orm2 bands (the fourth group) in each lane (b) were analyzed and plotted in the graph shown in c. (d, e) Exogenous PHS induces rapid Orm2 dephosphorylation. Wild-type cells expressing 3XFLAG-Orm2 from its endogenous locus were cultured in media containing methanol alone (mock) or 5 μM PHS dissolved in methanol for the indicated times. (f) Exogenous sphingosine (Sph) does not affect Orm2 phosphorylation. Wild-type cells expressing 3XFLAG-Orm2 from its endogenous locus were cultured in the presence of 5 μM Sph (dissolved in methanol) for the indicated times. (g, h) Exogenous PHS induces rapid Orm2 dephosphorylation in *lcb3Δ* cells or in *lcb4Δ lcb5Δ* cells. *lcb3Δ* cells or *lcb4Δ lcb5Δ* cells expressing 3XFLAG-Orm2 from its endogenous locus were cultured in the presence of 5 μM PHS for the indicated times.

response to a 10-min treatment at 25°C with a natural yeast sphingoid base, phytosphingosine (PHS), in a concentration-dependent manner. PHS at 5 μM is sufficient to cause maximal effects on Orm2 dephosphorylation (Figure 2c), whereas 20 μM of exogenous dihydrosphingosine (DHS), another sphingoid base, had a similar effect on Orm2 dephosphorylation (unpublished data). Dephosphorylated Orm2 greatly increased within 2 min of treatment with 5 μM PHS at 25°C (Figure 2, d and e). In contrast, adding 5 μM sphingosine (Sph), which is a mammalian sphingoid base that cannot rescue a yeast sphingolipid deficiency (Wells and Lester, 1983), did not affect Orm2 phosphorylation (Figure 2, d and f), indicating that the effect of PHS is highly specific. Furthermore, stearylamine (Jenkins and Hannun, 2001), which is a long-chain primary amine, also did not induce Orm2 dephosphorylation (unpublished data), providing further evidence for chemical specificity.

The rapid response to the exogenous addition of PHS (Figure 2e) suggests that the sphingoid base itself, rather than its downstream sphingolipid metabolites (Nagiec *et al.*, 1997; Figure 1), triggers Orm2 dephosphorylation. To explore this possibility further, we used a knockout mutant of the *LCB3* gene, which encodes the sphingosine-1P phosphatase required for ceramide synthesis from exogenous PHS (Mao *et al.*, 1997, 1999; Qie *et al.*, 1997; Figure 1). In the absence of Lcb3, exogenous PHS cannot be incorporated into ceramides (Qie *et al.*, 1997; Funato *et al.*, 2003). However, exogenous PHS still rapidly induced Orm2 dephosphorylation in the *lcb3Δ* strain (Figure 2g), suggesting that exogenous PHS induces Orm2 dephosphorylation without being converted to ceramides. A previous study indicated that lack of Orm2 results in increased PHS but decreased ceramides in vivo (Han *et al.*, 2010). Here we found that Orm1 phosphorylation is greatly reduced in a strain lacking Orm2 (Supplemental Figure S1a), in agreement with PHS being sufficient to induce Orm dephosphorylation. In addition, exogenous PHS still triggered Orm2 dephosphorylation in mutant cells lacking both Lcb4 and Lcb5 (Figure 2h), which are sphingoid base kinases required for phosphorylation of exogenously provided PHS (Nagiec *et al.*, 1998; Funato *et al.*, 2003; Figure 1). Thus, exogenous PHS itself, without being converted to phosphorylated sphingoid bases (Figure 1), can induce Orm2 dephosphorylation. However, compared with the wild-type cells (Figure 2e), Orm2 dephosphorylation in *lcb4Δ lcb5Δ* cells responds to PHS to a lesser extent (Figure 2h), suggesting that phosphorylated sphingoid bases may also contribute to Orm2 phosphoregulation.

Taken together, our data demonstrate that exogenous addition of PHS is sufficient to rapidly induce Orm2 dephosphorylation, indicating that Orm phosphorylation status is highly responsive to levels of sphingoid bases. Although we cannot exclude the possibility that ceramide and complex sphingolipids also

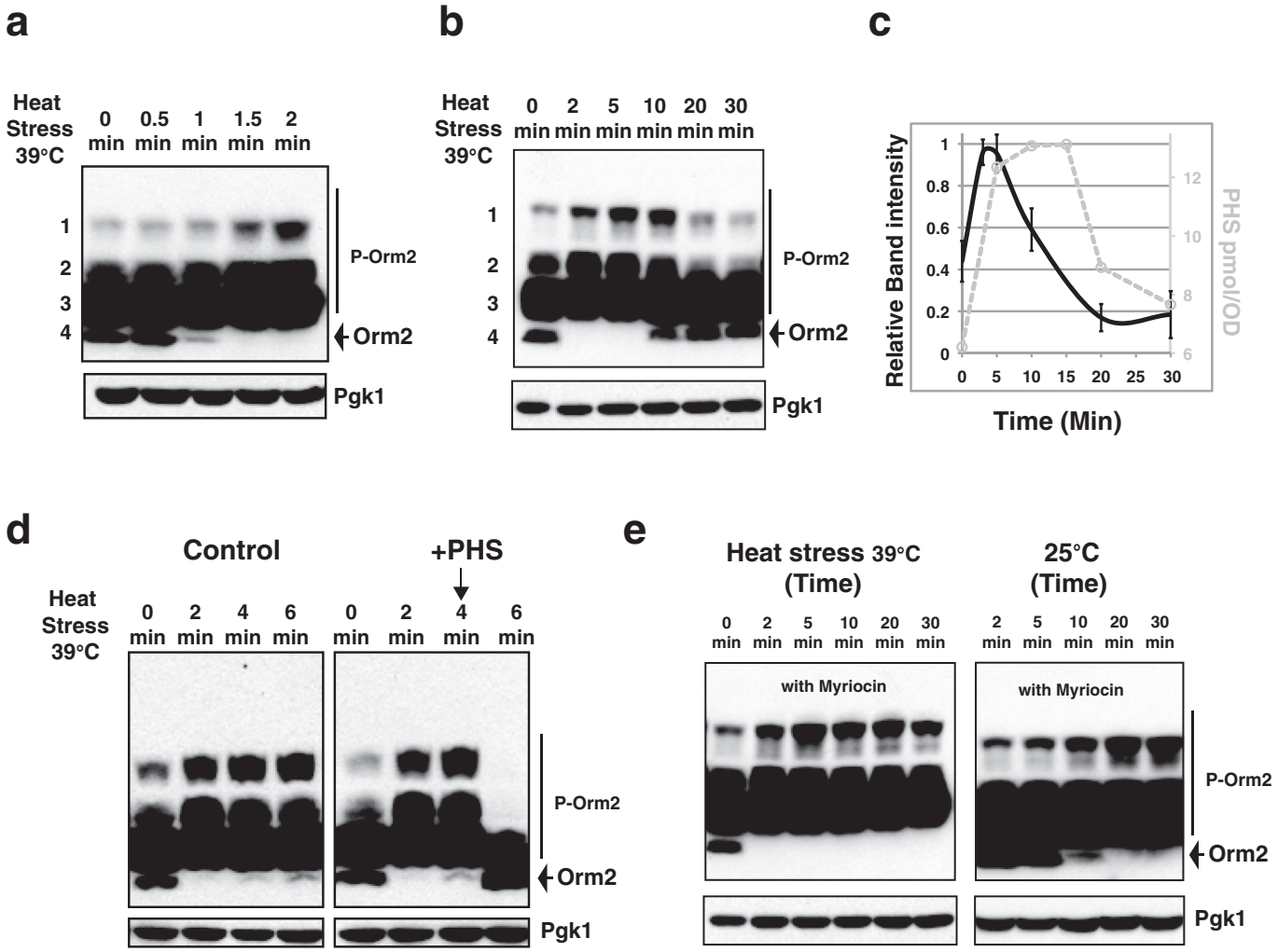

FIGURE 3: Orm2 phosphorylation dynamics and sphingoid base production upon heat shock. (a, b, d, e) Western blots showing Orm2 phosphorylation patterns after separation of the indicated yeast cell extracts on phosphate-affinity gels (top). P-Orm2 indicates phosphorylated forms of Orm2. Pgk1 was used as a loading control (bottom). Wild-type cells expressing 3XFLAG-Orm2 from its endogenous locus were used in all experiments and cultured to early log phase at 25°C before the indicated treatments. Cell extracts were prepared as described in *Materials and Methods*. (a–c) Temporal association between Orm phosphorylation dynamics and sphingoid base production upon heat stress. Cell extracts were prepared after cell cultures were shifted from 25 to 39°C for the indicated times. The relative intensity of the two slowest-migrating 3XFLAG Orm2 bands (groups 1 and 2) shown in b was quantified. Time-course experiments shown in b were performed an additional four times. Data collected from the five independent experiments were plotted in the graph shown in c (black line). Data shown represent the means with standard deviations. The C18-PHS concentration (c, gray, broken line) was determined by HPLC as described in *Materials and Methods*. (d), Orm2 phosphorylation status upon heat stress in the presence of exogenous sphingoid bases. Cells were shifted from 25 to 39°C for the indicated times. Methanol alone (control) or 5 μM PHS dissolved in methanol was added exogenously 4 min after cells were shifted from 25 to 39°C. (e) Orm2 phosphorylation upon heat stress in the presence of myriocin. Left, 0.5 μg/ml myriocin was added to cells upon shift from 25 to 39°C. Right, cells were treated with 0.5 μg/ml myriocin at 25°C for the indicated times.

contribute to Orm phosphoregulation, the results with *lcb3Δ* mutants (Figure 2g) support the conclusion that neither of these sphingolipids is required for Orm phosphoregulation.

Temporal association between Orm phosphorylation dynamics and de novo sphingoid base production in response to heat stress

Previous studies suggested that heat stress induces rapid transient de novo accumulation of sphingoid intermediates, including PHS. We next asked whether and how Orm protein phosphorylation may be involved in heat-induced sphingolipid biosynthesis.

We monitored Orm2 phosphorylation after cells were shifted from 25 to 39°C. Within 1 min after the temperature shift, dephosphorylated Orm2, which is the fastest-migrating band on phosphate-affinity gels, started to disappear (Figure 3a). Correspondingly, Orm2 phosphorylation levels increased, as evidenced by the increased abundance of the slower-migrating bands in the top two groups (Figure 3a). Orm2 phosphorylation further increased at 2 min (Figure 3a), with the maximal effect observed at ~5 min, followed by a decrease (Figure 3, b and c). Orm2 phosphorylation was further reduced by 20 min of heat stress and then stabilized (Figure 3, b and c). Several independent studies demonstrated

that heat stress induces rapid de novo accumulation of various sphingoid intermediates, including sphingoid bases (Dickson *et al*., 1997; Jenkins *et al*., 1997; Jenkins, 2003; Wells *et al*., 1998; Mao *et al*., 1999; Skrzypek *et al*., 1999). The sphingoid base levels peak by 10–15 min and decrease to near-basal levels by 30 min of heat stress (Dickson *et al*., 1997; Jenkins *et al*., 1997). Our measurements of PHS levels confirmed these previous conclusions (Figure 3c, gray, broken line). Thus, our results demonstrate a striking temporal association between Orm phosphorylation dynamics and de novo sphingoid base production in response to heat stress (Figure 3c).

High levels of Orm phosphorylation induced by heat stress decrease when the sphingoid bases (PHS) reach their peak levels (Figure 3c, time points between 5 and 15 min), suggesting that heat-induced sphingoid base accumulation and Orm dephosphorylation may be mechanistically linked. To investigate this possibility further, we altered the timing of the sphingoid base accumulation by adding exogenous PHS 4 min after the temperature shift, when de novo–synthesized sphingolipids normally just begin to accumulate (Figure 3c; Dickson *et al*., 1997; Jenkins *et al*., 1997; Wells *et al*., 1998; Mao *et al*., 1999; Skrzypek *et al*., 1999). In control cells, increased Orm2 phosphorylation peaks after 4 min of heat stress and is only slightly diminished after 6 min of heat stress (Figure 3d, left). However, addition of exogenous PHS at 4 min reduced Orm2 phosphorylation to the basal level at 6 min (Figure 3d, right).

To further test whether de novo synthesis of sphingoid intermediates in response to heat stress is required to trigger Orm dephosphorylation, we monitored Orm2 phosphorylation in response to heat stress in the presence of myriocin, a potent SPT inhibitor (Sun *et al*., 2000; Figure 1). No obvious changes in Orm2 phosphorylation were observed up to 5 min after myriocin treatment at 25°C (Figure 3e), and 2 min of heat stress induced Orm2 phosphorylation irrespective of myriocin presence (Figure 3, b and e), indicating that heat stress affects Orm2 phosphorylation before the myriocin-sensitive step. Of interest, in the presence of myriocin, the increased Orm2 phosphorylation did not decline even after 30 min of heat stress (compare Figure 3e with Figure 3b), indicating that de novo synthesis of sphingoid intermediates is required for reducing the increased Orm2 phosphorylation caused by heat stress. Together, these results provide strong evidence that increased levels of sphingoid intermediates trigger Orm dephosphorylation during the heat stress response. Thus, our results suggest that both exogenously provided sphingoid intermediates (such as PHS) and the accumulation of physiologically induced sphingoid intermediates (by heat stress), cause rapid Orm2 dephosphorylation (Figures 2 and 3).

Ypk kinase transmits heat stress signals to the sphingolipid biosynthesis pathway via Orm phosphorylation

Another example of a striking temporal association between Orm phosphorylation and sphingolipid intermediate production is the observation that heat stress triggers Orm phosphorylation in <1 min (Figure 3a), with phosphorylation reaching its maximum before the level of sphingoid bases reaches its peak (Figure 3, b and c). This temporal association raises the important question of determining how the heat stress signal is transmitted to induce rapid Orm phosphorylation.

A recent study suggested that Ypk1, a homologue of mammalian serum- and glucocorticoid-inducible kinase (Casamayor *et al*., 1999), specifically phosphorylates Orm2 residues S46, S47, and S48 in vitro (Roelants *et al*., 2011). To examine the role of phosphorylation of these serine residues on Orm function, we mutated S46, S47, and S48 of Orm2 to alanine or aspartic acid to generate *orm2-3A* (mimicking the dephosphorylated form) or *orm2-3D* (mimicking the phosphorylated form), respectively. The level of sphingoid bases was greatly reduced in *orm2-3A* cells and was greatly increased *orm2-3D* cells (Supplemental Figure S3), providing evidence that phosphorylation of these serines plays a role in sphingolipid production. Furthermore, as shown in Figure 4a, the majority of orm2-3A protein is in dephosphorylated forms, demonstrating that phosphorylation of Orm2 residues S46, S47, and S48 is responsible for the slower migration of phosphorylated Orm2 (Figure 4a; Breslow *et al*., 2010). Together, these results strongly suggest that phosphorylation of Orm2 by Ypk1 is involved in Orm-mediated sphingolipid biosynthesis regulation.

To determine whether the rapid Orm2 phosphorylation induced by heat stress (Figure 3c, time points between 0 and 4 min) requires Ypk kinase activity in vivo, we generated an analogue-sensitive (Bishop *et al*., 2000) *ypk1-as* allele in *ypk2Δ* background. Deletion of *YPK2* has no apparent phenotypic defect, but loss of *YPK1* results in slow growth (Chen *et al*., 1993). Knocking out both *YPK1* and *YPK2* causes lethality (Chen *et al*., 1993). Orm2 phosphorylation in response to heat stress in the *ypk1-as ypk2Δ* mutant was monitored in the presence of 3-MOB-PP1, which specifically inhibits ypk1-as kinase activity. Heat stress–induced phosphorylation no longer occurs when Ypk kinase activity is abolished (Figure 4b and Supplemental Figure S4a), indicating that heat stress facilitates Orm phosphorylation through Ypk kinase activity. More important, heat induced-sphingoid base accumulation no longer occurs when Ypk kinase activity is inhibited (Figure 4c and Supplemental Figure S4b). These results not only provide strong evidence that Orm phosphorylation positively regulates sphingolipid production in vivo, but they also indicate that Ypk kinase transmits the heat stress signals to sphingoid intermediate production through phosphorylation of the Orm proteins.

Ypk1/2 are phosphorylated and activated by Pkh1/2, the homologues of mammalian PKD1 (Casamayor *et al*., 1999). Neither Pkh1 nor Pkh2 alone is required for cell growth, but loss of both proteins causes lethality (Casamayor *et al*., 1999). Consistently, heat stress–induced phosphorylation is abolished when Pkh kinase activity is inhibited (Figure 4d).

A recent study proposed that Orm2 expression levels regulate sphingolipid synthesis (Liu *et al*., 2012). However, as shown in Figure 3a, Orm phosphorylation starts to increase within 1 min after introduction of heat stress, reaching its maximum at around 5 min, and then decreases. The rapid time scale of these events strongly suggests that regulation of sphingolipid synthesis in response to heat is primarily due to the changes in Orm phosphorylation, not to changes in Orm expression.

Together, the results shown in Figures 3 and 4 establish a physiologically relevant context for the association between sphingolipid homeostasis and Orm protein phosphorylation dynamics and identify a novel feedback pathway for temporal regulation of sphingolipid biosynthesis during the heat stress response: heat stress rapidly activates the Pkh-Ypk signaling pathway to induce Orm phosphorylation, which in turn promotes sphingolipid intermediate production. The accumulated sphingoid intermediates then trigger Orm dephosphorylation, which in turn down regulates sphingolipid biosynthesis.

Cdc55-PP2A is a key phosphatase that counteracts Ypk1 activity in Orm-mediated sphingolipid biosynthesis regulation

Orm phosphorylation state is set not only by kinases but also by phosphatases. We next sought to identify the phosphatase involved in Orm dephosphorylation. We hypothesized that the phosphatase acting on the Orm proteins is likely to be involved in

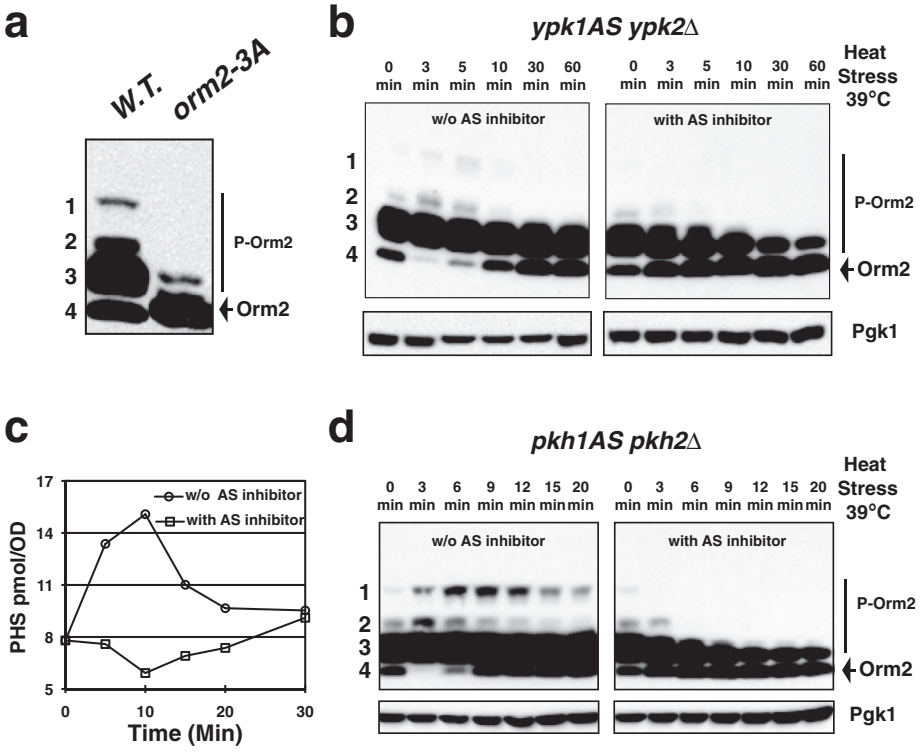

FIGURE 4: Ypk kinase transmits heat stress signals to the sphingolipid biosynthesis pathway via Orm phosphorylation. (a, b, d) Western blots showing Orm2 phosphorylation patterns after separation of the indicated yeast cell extracts on phosphate affinity gels (top). P-Orm2 indicates phosphorylated forms of Orm2. Pgk1 was used as a loading control (bottom). All cells were grown to early log phase in YPD at 25°C before the indicated treatments. Cell extracts were prepared as described in *Materials and Methods*. (a) Orm2 residues S46, S47, and S48 are responsible for slower migration of phosphorylated Orm2. (b) Orm2 phosphorylation in response to heat stress in the absence of Ypk activity. Dimethyl sulfoxide (DMSO) alone or 50 μM of 3-MOB-PP1 dissolved in DMSO, which specifically inhibits ypk1-as kinase activity, was added to *ypk1as ypk2Δ* cells upon shift from 25 to 39°C. (c) Heat-induced sphingoid base accumulation requires Ypk kinase activity. The C18-PHS concentration was determined in the *ypk1as ypk2Δ* cells cultured in the absence (line with circle) or the presence (line with square) of 50 μM 3-MOB-PP1 shifted from 25 to 39°C for the indicated times. (d) Orm2 phosphorylation status upon heat stress in the absence of Pkh activity. DMSO alone or 100 μM CZ21 dissolved in DMSO, which specifically inhibits pkh1-as kinase activity, was added to *pkh1as pkh2Δ* cells upon shift from 25 to 39°C.

and/or regulated by sphingolipid levels. PP2A (Figure 5a) was identified as an attractive candidate for a ceramide-activated protein phosphatase in yeast (Nickels and Broach, 1996). Inactivation of Cdc55 or the catalytic subunits of PP2A was reported previously to suppress the endocytic defects of a mutant with impaired SPT activity (Friant *et al.*, 2000), suggesting that PP2A may be involved in sphingolipid production. We explored the specific relationship between Cdc55-PP2A activity and Orm regulation at standard growth temperatures (25 or 30°C).

The yeast PP2A, similar to its mammalian counterparts, is a heterotrimer composed of three distinct subunits, namely A (the structural subunit, encoded by *TPD3*), B (the regulatory subunit, encoded by two distinct genes, *CDC55* and *RTS1*), and C (the catalytic subunit, also encoded by two distinct genes, *PPH21* and *PPH22*) (Jiang, 2006). If PP2A does dephosphorylate Orm2, lack of PP2A activity would be expected to result in increased Orm2 phosphorylation. *tpd3Δ* and *pph21Δ pph22Δ* showed increases in both phosphorylated (the two slowest-migrating bands) and dephosphorylated forms (the fastest-migrating band) of Orm2 (Figure 5b). The total Orm2 expression levels in these two mutants also appeared to be much higher than in wild-type cells (Figure 5b). Moreover, both of these strains have severe growth defects (Figure 5c). Thus, we conclude that it is not possible to address PP2A function, particularly in Orm phosphoregulation, using these two mutants; the elevated Orm2 expression in these mutants may affect sphingolipid synthesis (Liu *et al.*, 2012). In contrast, *pph21Δ*, *pph22Δ*, *cdc55Δ*, and *rts1Δ* single mutants grow relatively normally (Figure 5c). Compared to wild-type cells, Orm2 phosphorylation levels increased dramatically in *cdc55Δ* mutants (Figure 5b). *pph21Δ* cells also showed higher Orm2 phosphorylation (Figure 5b). In contrast, the Orm2 phosphorylation patterns in *rts1Δ* and wild-type cells were indistinguishable (Figure 5b). These results indicate that Cdc55-PP2A activity, rather than Rts1-PP2A activity, mediates Orm dephosphorylation.

We next examined how PP2A mutants respond to the SPT inhibitor myriocin. Previous studies suggested that Orm proteins negatively regulate SPT activity and that Orm phosphorylation relieves their inhibition of SPT (Breslow *et al.*, 2010; Figure 1). Thus, impaired PP2A activity, which increases Orm2 phosphorylation, might lead to enhanced myriocin resistance. Unfortunately, *tpd3Δ* and *pph21Δ pph22Δ* mutants show severe growth defects even without myriocin (Figure 5c), making assessment of their myriocin sensitivity difficult. However, *pph21Δ*, *pph22Δ*, and *cdc55Δ* single mutants showed resistance to 0.75 μg/ml myriocin, a concentration that inhibits growth of wild-type cells (Figure 5c). The same set of PP2A mutants could not survive 1 μg/ml myriocin (Figure 5c), a concentration that likely causes more complete inhibition of SPT activity. In addition, *cdc55Δ* rescued the growth of the *lcb1-100 ts* mutant at a semirestrictive temperature but not at the restrictive temperature (Figure 5d). Lcb1 is an essential component of yeast SPT (Buede *et al.*, 1991). Thus, it appears that the myriocin resistance of the Cdc55-PP2A–deficient mutants is only observable when SPT activity is not completely blocked and thus is still regulated by Orm proteins. The *rts1Δ* mutant shows resistance to even 1 μg/ml myriocin (Figure 5c), implying that the *rts1Δ* mutant cells and *cdc55Δ* mutant cells are resistant to myriocin through a different mechanism.

To further explore the relationship between PP2A, the Orm proteins, and SPT regulation, we examined the myriocin sensitivity of PP2A mutants in an *orm*-null mutant background. We found that *pph21Δ orm1Δ orm2Δ* and *cdc55Δ orm1Δ orm2Δ* triple mutants were not resistant to 0.75 μg/ml myriocin (Figure 6, a and b), whereas *rts1Δ orm1Δ orm2Δ* mutants were resistant to even 1 μg/ml myriocin (Figure 6a), establishing that Orm proteins are required for the myriocin resistance of Cdc55-PP2A–deficient mutants but not of the *rts1Δ* mutant.

We next tested the role of Ypk phosophorylation of the three Orm2 serines (Roelants *et al.*, 2011) in the myriocin resistance of

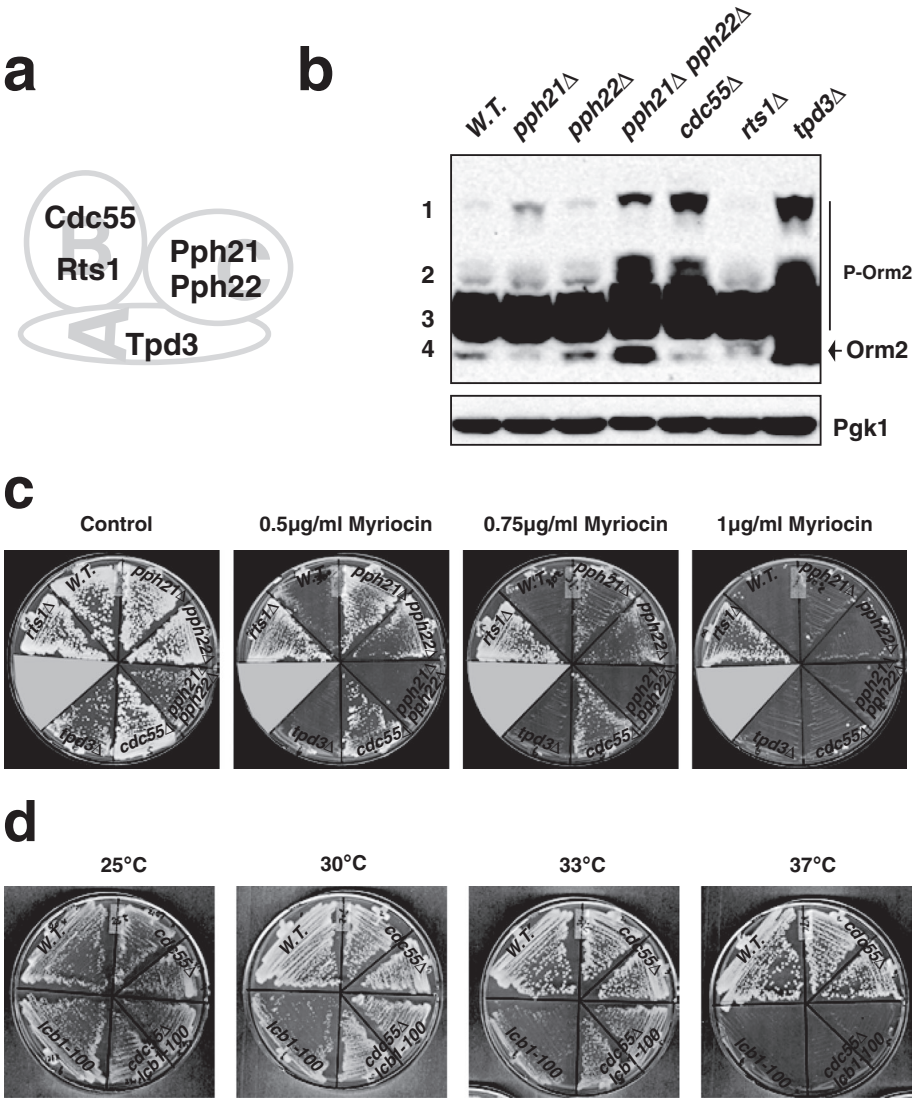

FIGURE 5: Orm2 phosphorylation status and myriocin resistance in various PP2A mutants. (a) Subunit composition of yeast PP2A. (b) Orm2 phosphorylation status in cells lacking the indicated PP2A subunits. Western blots showing the phosphorylation patterns for Orm2 after separation on phosphate-affinity gels. All cells were grown to early log phase in YPD at 25°C. Cell extracts were prepared as described in *Materials and Methods*. Pgk1 was used as a loading control. (c) Growth of PP2A mutants in the presence of myriocin. Various mutants were grown on plates containing the indicated concentrations of myriocin at 30°C for 3 d. (d) Cdc55 absence rescued the growth of an *lcb1-100 ts* mutant at semirestrictive temperature but not restrictive temperature. Mutants were grown at the indicated temperature for 3 d. Note that *cdc55Δ lcb1-100* cells grow better than *lcb1-100* at 30 and 33°C.

Cdc55-PP2A deficient mutants. As shown in Figure 6, b and d, the *pph21Δ orm2-3D orm1Δ* and *cdc55Δ orm2-3D orm1Δ* strains show myriocin resistance, whereas the *pph21Δ orm2-3A orm1Δ* and *cdc55Δ orm2-3A orm1Δ* strains do not. These results support the conclusion that Cdc55-PP2A counteracts Ypk1 phosphorylation of the three indicated Orm2 serines in regulation of sphingolipid production. In contrast, the *rts1Δ orm2-3A orm1Δ* strain is resistant to myriocin (Figure 6d), which further confirms our conclusion that myriocin resistance of *rts1Δ* is independent of the Orm proteins.

A previous study demonstrated that *cdc55Δ* confers resistance to rapamycin (Jiang and Broach, 1999), which is an inhibitor of TOR signaling. Strikingly, we found that Orm proteins are required for myriocin resistance of *cdc55Δ* cells but not for their rapamycin resistance (Figure 6c). This result establishes that whereas PP2A may be involved in multiple cellular signaling pathways (Jiang, 2006), Cdc55-PP2A and Orm protein activities are linked specifically for sphingolipid biosynthesis. Furthermore, sphingoid base levels were substantially increased in *cdc55Δ* cells (Supplemental Figure S3), indicating that Cdc55-PP2A activity is involved in sphingolipid production.

PP2A activity contributes to the regulation of Orm phosphorylation dynamics in response to heat stress

We next examined the role of Cdc55-PP2A in Orm2 dephosphorylation dynamics in response to heat stress. We generated a *PP2A ts* mutant, *pph21 E102K pph22Δ* (Lin and Arndt, 1995), which allowed us to simultaneously introduce heat stress and turn off PP2A activity. As shown in Figure 7, substantially less dephosphorylated Orm2, seen as the fastest-migrating bands on a phosphate-affinity gel, appears in the *PP2A ts* mutant at both the 10- and 15-min time points compared with *pph22Δ* cells. The observation that Orm dephosphorylation was not completely abolished in the *PP2A ts* mutant suggests that other phosphatases may also contribute to Orm protein dephosphorylation in response to heat stress. It is also possible that PP2A activity was not completely lost in the *PP2A ts* mutant after the short temperature shift. Nevertheless, our results indicate that PP2A activity contributes to regulation of Orm phosphorylation dynamics in response to heat stress.

DISCUSSION

A model for how Orm phosphorylation regulation controls sphingolipid biosynthesis in response to stress in a kinetically coupled manner

As bioactive molecules, sphingoid intermediates transmit signals when their cellular levels change in response to various stresses (Hannun and Obeid, 2008; Dickson, 2010; Nikolova-Karakashian and Rozenova, 2010). However, both lack of sphingolipids and constitutive high levels of sphingolipids compromise cell viability (Buede *et al.*, 1991; Chung *et al.*, 2001). Thus, biosynthesis of sphingoid intermediates in response to stresses requires precise temporal control. Previously, very little was known about the regulatory mechanism.

Here we provided evidence supporting a model in which the conserved Pkh-Ypk signaling cascade and Cdc55-PP2A facilitate/ensure rapid, transient sphingolipid production upon heat stress through regulation of Orm protein phosphorylation (Figure 8): 1) A Pkh-Ypk cascade is rapidly activated upon heat stress. 2) Orm phosphorylation rapidly increases. 3) Orm phosphorylation releases inhibition of SPT activity. 4) Activated SPT promotes de novo synthesis of sphingoid intermediates. 5) Accumulated sphingoid intermediates act as signaling molecules to initiate the cellular heat shock

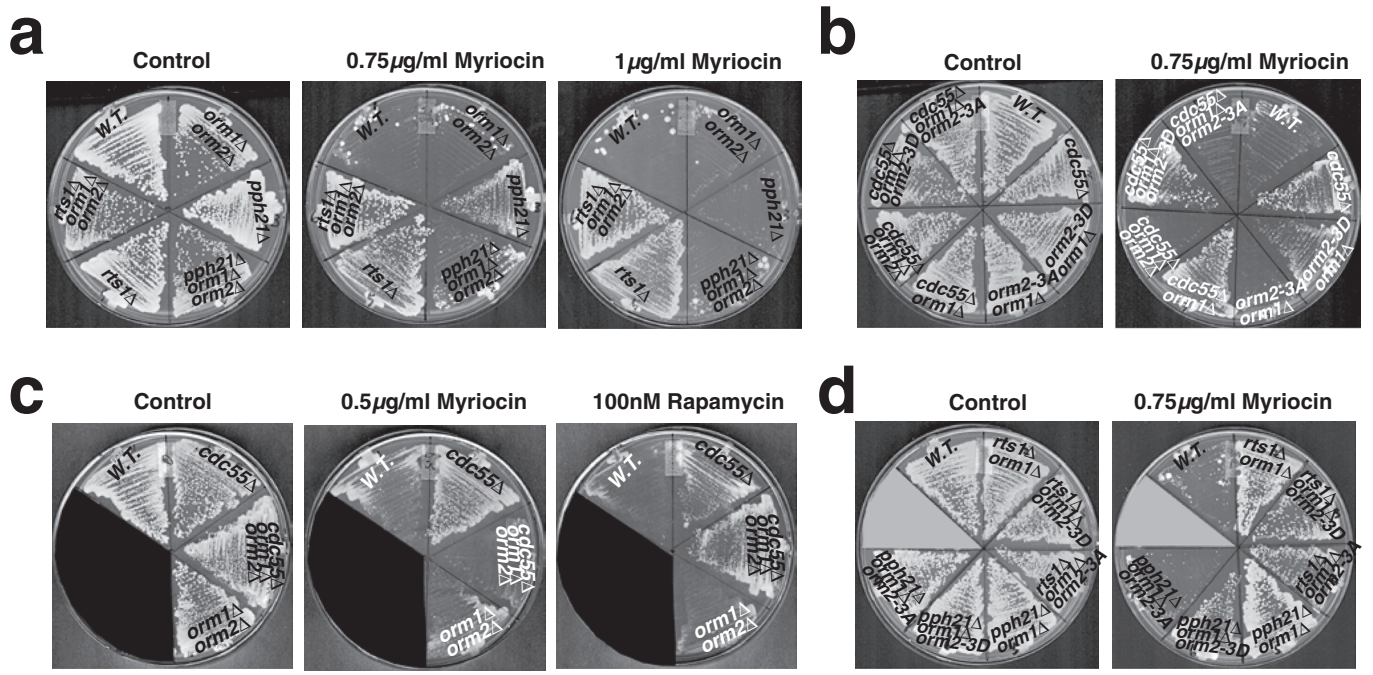

FIGURE 6: PP2A-deficient mutants confer myriocin resistance specifically through effects on the Orm proteins. (a, b, d) Impaired Cdc55-PP2A activity results in myriocin resistance dependent on Orm phosphorylation status. Various mutants were grown at 30°C for 3 d on plates containing the indicated myriocin concentrations. (c) Orm proteins are required for myriocin resistance but not rapamycin resistance in *cdc55Δ*. Various mutants were grown on plates containing 0.5 μg/ml myriocin or 100 nM rapamycin at 30°C for 3 d.

responses, as described in the *Introduction*. Sphingoid intermediate accumulation also feeds back to dephosphorylate Orm proteins, possibly by inhibiting Pkh-Ypk kinase activity or/and activating Cdc55-PP2A phosphatase activity. 6) Dephosphorylated Orm proteins inhibit SPT activity.

The Pkh-Ypk cascade was previously proposed to be downstream of heat-induced sphingolipid accumulation (Dickson, 2010). However, our data indicate that the Pkh-Ypk cascade is activated in response to heat stress and induces de novo sphingolipid biosynthesis (Figure 4). Of interest, a previous in vitro study indicated that Pkh1/Pkh2 kinases are inhibited by micromolar concentrations of sphingoid bases (Friant *et al.*, 2001). Thus, the most harmonious interpretation of our results and previous findings is that the Pkh-Ypk cascade is also regulated by heat-induced sphingolipid intermediate accumulation via a negative feedback mechanism by which increased sphingolipids inhibit Pkh-Ypk kinase–mediated phosphorylation of Orm proteins, thereby restoring SPT inhibition.

How could heat stress activate Ypk kinases to trigger Orm2 phosphorylation in such a rapid manner (Figure 3a)? Breslow *et al.* (2010) showed that disruption of sphingolipid biosynthesis by myriocin results in an increase in Orm phosphorylation. However, the increase in Orm phosphorylation in response to heat stress apparently is not due to low levels of sphingolipids, because both the levels of Orm phosphorylation and the levels of sphingolipid intermediates actually continue to increase at these early time points after heat stress (Figure 2C, time points between 0 and 4 min; Dickson *et al.*, 1997; Jenkins *et al.*, 1997; Jenkins, 2003; Wells *et al.*, 1998; Mao *et al.*, 1999; Skrzypek *et al.*, 1999). Moreover, our data suggest that heat stress affects Orm phosphorylation before the myriocin-sensitive step (Figure 3e). Ypk1 activation is known to require its phosphorylation by both Pkh kinases and the TORC2 complex (Casamayor *et al.*, 1999; Kamada *et al.*, 2005). Recently Slm1/2 was demonstrated to play important roles in recruiting Ypk1 to the plasma membrane for activation by the TORC2

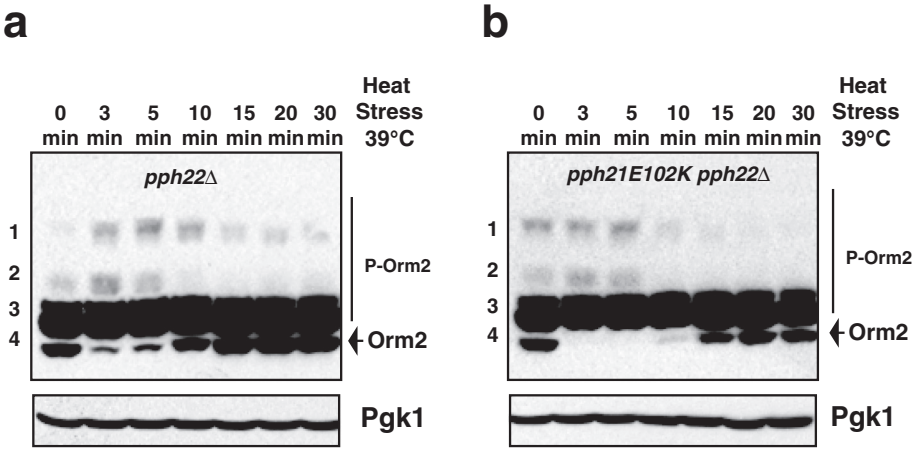

FIGURE 7: Orm phosphorylation status in PP2A-deficient mutants upon heat shock. Western blots showing the phosphorylation patterns for Orm2 after separation on phosphate-affinity gels. P-Orm2 indicates phosphorylated forms of Orm2. Pgk1 was used as a loading control. Cell extracts were prepared from cells shifted from 25 to 39°C for the indicated times as described in *Materials and Methods*. The *pph21E102K pph22Δ* mutant is temperature sensitive.

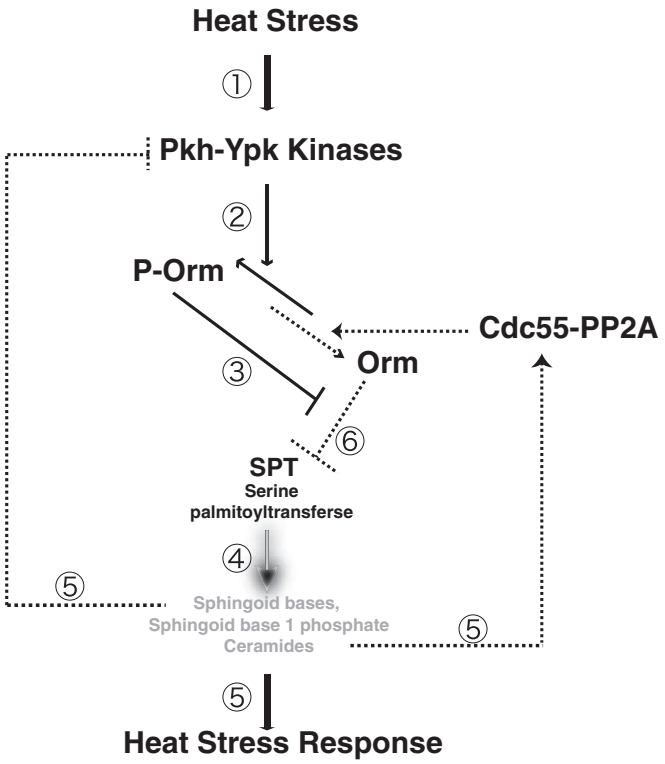

FIGURE 8: A feedback regulation pathway in which Orm protein phosphorylation dynamics rapidly and precisely regulate sphingolipid biosynthesis in response to heat stress. See the text for a description.

complex (Niles *et al.*, 2012). Of interest, both Pkh kinases and Slm1/2 localize in punctate eisosome plasma membrane domains (Walther *et al.*, 2007; Grossmann *et al.*, 2008), and the TORC2 complex localizes in small foci on the plasma membrane but not in eisosomes (Berchtold and Walther, 2009). Another recent study suggested that plasma membrane stress might induce relocalization of Slm1/2 and activation of TORC2 (Berchtold *et al.*, 2012). Thus, it is possible that heat stress may rapidly decrease the rigidity of plasma membrane, resulting in release of Pkh kinase and Slm1/2 from eisosomes, thereby triggering Ypk kinase activation.

Both the lipids and the proteins involved in this regulatory circuit (Figure 8) are highly conserved between yeast and mammalian cells. Thus, the mechanism we proposed here may serve a general basis for how Orm phosphoregulation controls sphingolipid biosynthesis in response to stress in a kinetically coupled manner.

Orm phosphorylation status is highly responsive to sphingoid bases

In this study, we demonstrated that the high levels of Orm phosphorylation induced by heat stress decline while the sphingoid bases (PHS) reach their peak levels (Figure 3c, time points between 5 and 15 min). Several previous studies established that sphingoid bases and sphingoid base phosphates accumulate with similar timing (Dickson *et al.*, 1997; Jenkins *et al.*, 1997). However, the heat-induced ceramides peak ~10 min after the sphingoid bases reach their peak (Dickson *et al.*, 1997; Jenkins *et al.*, 1997), and no obvious changes in the levels of complex sphingolipids were observed during 2 h of heat stress (Jenkins *et al.*, 1997). Thus, the decrease of Orm phosphorylation induced by heat stress is kinetically coupled to changes in sphingoid base and sphingoid base phosphate levels but not ceramide or complex sphingolipid levels, suggesting that Orm phosphorylation is primarily regulated by sphingoid bases and sphingoid base phosphates. In agreement with these conclusions, our experiments indicate that exogenously provided sphingoid bases are sufficient to induce Orm dephosphorylation without being converted to ceramide or to complex sphingolipids (Figure 2).

A recent study proposed that Orm1 and Orm2 may regulate sphingolipid synthesis via two different mechanisms (Liu *et al.*, 2012). We observed the phosphorylation dynamics of Orm1 and Orm2 upon heat stress to be very similar (unpublished data), suggesting that both of the Orm proteins function similarly in regulation of the sphingolipid biosynthesis response to heat stress. However, we found that Orm2 expression was at least 10 times higher than Orm1 expression (Supplemental Figure S1a). It is also worth mentioning that neither C-terminally tagged Orm1 nor Orm2 is functional (unpublished data) and that Orm1 tagged at its N-terminus with 3XFLAG cannot fulfill Orm1 function in the *orm2Δ* background (Supplemental Figure S1A). Our studies indicate that Orm function is preserved in N-terminally tagged Orm2.

Phosphate-affinity gels separated Orm2 protein into four mobility groups. Western blotting analysis revealed that the majority of Orm proteins are moderately phosphorylated (bands in the third group, Figure 2a) under standard growth conditions. This large pool of moderately phosphorylated Orm protein may explain how cells rapidly respond to various environmental stresses in a graded manner. In addition, the majority of orm2-3A proteins were detected as dephosphorylated forms, and levels of sphingoid bases were greatly reduced in *orm2-3A* cells, further supporting the conclusion that dephosphorylated Orm proteins negatively regulate sphingolipid biosynthesis. Given that the reduction of dephosphorylated Orm2 and the increase in hyperphosphorylated Orm2 occur at the same time in response to heat stress (Figure 3b, time points 2 and 5 min), SPT activation in response to heat stress could be caused by release of SPT inhibition by dephosphorylated Orm2, by positive regulation of SPT by hyperphosphorylated Orm2, or both.

Cdc55-PP2A counteracts Ypk1 activity in Orm-mediated sphingolipid biosynthesis regulation

In comparison to kinases, phosphatases often function in a less specific manner. In this study, we performed a series of experiments to demonstrate a specific relationship between Cdc55-PP2A and the Orm proteins in regulating sphingolipid biosynthesis. First, sphingolipid biosynthesis increased in *cdc55Δ* mutants. Second, Orm proteins were required for myriocin resistance but not for rapamycin resistance of *cdc55Δ* cells. Finally, genetic analysis suggested that Cdc55-PP2A functions to counteract Ypk1 kinase–mediated Orm phosphorylation.

In contrast, we found that Rts1, another PP2A regulatory subunit, is involved in sphingolipid regulation in an Orm protein–independent manner. Previous studies suggested that Sac1 binds and modulates SPT activity in a pathway independent of Orm proteins (Breslow *et al.*, 2010). Future investigations are needed to test whether Rts1-PP2A is involved in Sac1 modulation of SPT activity.

PP2A was previously identified as an attractive candidate for a ceramide-activated protein phosphatase in yeast (Nickels and Broach, 1996), although direct evidence that ceramides (or other sphingoid intermediates) activate PP2A has not been reported. It is not clear whether other phosphatases are also involved in Orm phosphoregulation. Loss of Ypk kinase activity abolished sphingolipid biosynthesis in response to heat stress (Figure 4c), but Orm phosphorylation still decreased (Figure 4b, right), suggesting that Cdc55-PP2A may affect Orm phosphorylation at least partially in a constitutive manner. Because both Pkh kinases and the TORC2 complex are required for Ypk kinase activity, an alternative explanation for our

results could be that Cdc55-PP2A controls Orm phosphorylation by negatively regulating these two upstream effectors of Ypk kinases, instead of directly dephosphorylating the Orm proteins. However, this possibility seems unlikely because Orm2 dephosphorylation should have no longer occurred upon loss of Ypk kinase activity (Figure 4b, right), which is contrary to our observation.

MATERIALS AND METHODS

Media and strain construction

The yeast strains used in this study are listed in Supplemental Table S1. They were grown in standard rich media (yeast extract/peptone/dextrose [YPD]). The primers used for cloning are listed in Supplemental Table S2.

To generate a strain expressing the 3XFLAG-fused Orm2 protein from its genetic locus, we first constructed a vector using following strategies. Two DNA fragments were obtained by PCR amplification, using primer pairs of *Hin*dIII-ORM2-F/FLAG-ORM2-interR and FLAG-ORM2-interF/*Xho*I-ORM2-R, respectively. The two DNA fragments were then converted into one by PCR amplification, using primer pairs of *Hin*dIII-ORM2-F/*Xho*I-ORM2-R. The resulting PCR product was digested and ligated into the pBluescript II KS(+) vector using the *Hin*dIII and *Xho*I restriction enzymes. In addition, a *NAT1* (nourseothricin acetyltransferase) DNA fragment, amplified by primer pairs NatR-ORM2-F/NatR-ORM2-R, was inserted into the same vector using the *Hin*dIII and *Spe*I restriction enzymes. The resulting plasmid, named pBS-*NAT1-FLAG-ORM2*, was digested with *Hin*dIII and *Xho*I, and the fragment containing *NAT1-FLAG-ORM2* was transformed into a yeast strain in which *ORM2* had been replaced by *Candida glabrata* (*Cg*) *URA3*. We isolated transformants that no longer grow on a plate lacking uracil but do grow on a nourseothricin-dihydrogen sulfate (clonNAT)–containing plate. The pBS-NAT1-FLAG-ORM2 plasmid was also used as a template to generate *NAT1-FLAG-ORM2-3A* and *NAT1-FLAG ORM2-3D* plasmids using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) and primers ORM2-3A-F/ORM2-3A-R and ORM2-3D-F/ORM2-3D-R, respectively. A strain expressing 3XFLAG-Orm1 was generated by a similar strategy but using different primers, listed in Supplemental Table S2.

To obtain strains expressing Ypk1-as (Ypk1 L424A), two fragments were PCR amplified from genomic DNA using primers OYS227/OYS268 and OYS228/OYS267. The two fragments were then converted into one by PCR amplification, using primer pairs of OYS227/OYS228. The resulting fragment was digested and ligated into pRS306 vector. pRS306-*YPK1AS* (*YPK1 L424A*) was linearized using *Stu*I and transferred into the *URA3* locus of a *ypk1Δ::CgLEU2* strain.

To obtain strains expressing Pkh1-as, a fragment containing 500 base pairs upstream and downstream of *PKH1* was PCR amplified from genomic DNA using primers PKH1-*Xho*I-F and PKH1-*Sac*II-R. The PCR product was digested by *Xho*I/*Sac*II and was ligated into pRS306 vector, creating pRS306-*PKH1*. pRS306-*PKH1* was used as template to generate the *PKH1-AS* (*PKH1 F187V, L203A*) plasmid using the QuikChange Lightning Site-Directed Mutagenesis Kit and primers PKH1-F187V/L203A-F and PKH1-F187V/L203A-R. *PKH1-AS* (*PKH1 F187V, L203A*) plasmid was linearized using *Stu*I and inserted into the *URA3* locus of a *pkh1Δ::CgLEU2* strain.

PPH21 E102K plasmid was obtained using a similar strategy as for the *PKH1-AS* plasmid. Primers PPH21-*Bam*HI-F and PPH21-*Not*I-R were used to amplify the *PPH21* fragment. The *PPH21* fragment was ligated into the pRS306 vector, creating pRS306-*PPH21*. pRS306-*PPH21* was used as a template to generate *PPH21 E102K* plasmid using the QuikChange Lightning Site-Directed Mutagenesis Kit and primers PPH21-E102K-F and PPH21-E102K-R. Linearized pRS306-*PPH21 E102K* plasmid was integrated into the *URA3* locus of a *pph21Δ::CgHIS3* strain.

Lipid reagents

Phytosphingosine purified from *Saccharomyces cerevisiae* was obtained from Avanti Polar Lipids (Alabaster, AL). Sphingosine was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and stearylamine was obtained from Sigma-Aldrich (St. Louis, MO). All lipids were dissolved in methanol.

Preparation of whole-cell extracts

Yeast cells were grown to early logarithmic phase in YPD. After the indicated treatments, cold trichloroacetic acid (TCA) was added to the yeast culture to a final concentration of 20% (vol/vol). The growth medium was removed after centrifugation, and cell pellets were flash frozen in liquid nitrogen. The cells were thawed, resuspended in 5% (vol/vol) TCA, and lysed by bead beating at 4°C for 10 min. Whole-cell extracts were collected by 5 min of centrifugation at 14,000 × *g* at 4°C. The pellets were resuspended in SDS–PAGE sample buffer containing 50 mM dithiothreitol.

Detection of protein phosphorylation

To detect phosphorylation-dependent mobility shifts of FLAG-Orm1 and FLAG-Orm2, whole-cell extracts were loaded onto 8% SDS polyacrylamide gels containing 25 μM Phos-tag acrylamide (Wako Chemicals USA, Richmond, VA) and 25 μM $MnCl_2$. Before transfer to nitrocellulose membranes, gels were washed twice for 10 min in 2 mM EDTA containing transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% [vol/vol] methanol) and then once for 10 min in transfer buffer without EDTA. Membranes were then probed with 1:4000 mouse anti-FLAG (Sigma-Aldrich).

Lipid analysis by high-performance liquid chromatography

Extraction and processing of sphingoid bases from yeast cells for fluorescence high-performance liquid chromatography (HPLC) analysis using the AQC reagent (Waters, Milford, MA) was performed as described previously (Lester and Dickson, 2001). Sampling of heat-stressed cells was as described. HPLC analysis was performed using a C18 column (4.6 × 250 mm, XDB-C18; Hewlett-Packard, Palo, Alto, CA) on a Shimadzu LC10A series liquid chromatography system. Isocratic elution was carried out for 60 min at a flow rate of 1.0 ml/min. Lipid-reacted AQC reagent was excited with 244-nm UV radiation, and the resultant emission signal at 398 nm was detected. C18-PHS reacted with the AQC reagent was used as a standard for quantification.

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