Rom2-dependent Phosphorylation of Elo2 Controls the Abundance of Very Long-chain Fatty Acids*§

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**Background:** Sphingolipids are synthesized from very long-chain fatty acids and sphingoid bases.

**Results:** Rom2 controls Elo2 phosphorylation to regulate very long-chain fatty acid synthesis.

**Conclusion:** Distinct signaling pathways emanating from the plasma membrane regulate the different branches of sphingolipid synthesis.

**Significance:** Signaling from the plasma membrane regulates a key step in sphingolipid synthesis, required for lipid homeostasis of the plasma membrane.

Sphingolipids are essential components of eukaryotic membranes, where they serve to maintain membrane integrity. They are important components of membrane trafficking and function in signaling as messenger molecules. Sphingolipids are synthesized de novo from very long-chain fatty acids (VLCFA) and sphingoid long-chain bases, which are amide linked to form ceramide and further processed by addition of various headgroups. Little is known concerning the regulation of VLCFA levels and how cells coordinate their synthesis with the availability of long-chain bases for sphingolipid synthesis. Here we show that Elo2, a key enzyme of VLCFA synthesis, is controlled by signaling of the guanine nucleotide exchange factor Rom2, initiating at the plasma membrane. This pathway controls Elo2 phosphorylation state and VLCFA synthesis. Our data identify a regulatory mechanism for coordinating VLCFA synthesis with sphingolipid metabolism and link signal transduction pathways from the plasma membrane to the regulation of lipids for membrane homeostasis.

SLs are also capable of forming nano-domains with sterols in the membrane, which may organize proteins for cell signaling (e.g. from the plasma membrane) or for vesicular trafficking (e.g. from the trans-Golgi network) (5–9). In addition, several intermediates of SL metabolism, such as sphingosine 1-phosphate and ceramide, function as messenger signaling molecules within and between cells (10, 11).

Surprisingly little is known regarding the regulation of SL abundance in membranes. Much of the current knowledge is derived from studies of the yeast *Saccharomyces cerevisiae*, which has been an excellent model system to dissect the principles of SL regulation. Although there are substantial differences in SLs between yeast and mammals (e.g. in the lipid headgroups), general aspects of SL metabolism, including key enzymes and regulatory features, are evolutionarily conserved (12–16). As in metazoans, including humans, SL synthesis in yeast occurs in two branches that provide VLCFAs (saturated fatty acids containing 20–26 carbon atoms) and sphingoid long-chain bases. These two components are joined to form ceramides, which then gain headgroups consisting of inositol phosphate and mannose to form complex sphingolipids (11) (Fig. 1A). In yeast, the essential function of SLs appears to be mediated by the VLCFA component. Yeast that lack SLs can survive by complementation with mutations that incorporate VLCFAs into membrane phospholipids, where they are normally not found (17).

The abundance of the VLCFA and sphingoid long-chain bases must be balanced by cells to provide adequate substrates for SL synthesis. The first and rate-limiting step of sphingoid long-chain base synthesis is the condensation of serine with palmitoyl-CoA, catalyzed by serine palmitoyltransferase (SPT, encoded by *LCB1*, *LCB2*, and *TSC3* in yeast). When cells have sufficient levels of SLs, SPT activity is inhibited by formation of an inhibitory complex with Orm1 or Orm2 (Orm1/2 proteins). When SL levels fall in the plasma membrane, a signaling cascade initiated by Smn1/2 protein-mediated control of TORC2 leads to the phosphorylation of Orm1/2 through the interme-
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TABLE 1

Yeast strains used in this study

| Strain       | Genotype                               | Reference |
|--------------|----------------------------------------|-----------|
| TWY70        | MATa his3 leu2 lys2 ura3               | 56        |
|              | MATa elo2::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa elo3::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa fα38Δ::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa PH51::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa TSC1::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa rom2::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa sac7::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa bcl1::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa mkk2::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa mkk1::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
|              | MATa wsc1::KAN8 his3Δ1; leu2Δ0; ura3Δ0 met15Δ0 |          |
| TWY1050      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY2594      | MAT α his3 leu2 lys2 ura3 pV532_ypk2::URA3::URA3 | This study |
| TWY2595      | MAT α his3 leu2 lys2 ura3 pY352_ypk2::URA3::URA3 | This study |
| TWY2596      | MAT α his3 leu2 lys2 ura3 YCP50_pkc2::URA3::URA3 | This study |
| TWY3181      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3186      | MAT α ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3187      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3188      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3189      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3190      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3442      | MAT α his31 leu2Δ0 lys2Δ0 ura3Δ0 OMR1-HA::HIS | This study |
| TWY3446      | MAT α his31 leu2Δ0 lys2Δ0 ura3Δ0 SLT2-HA::HIS | This study |
| TWY3693      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3694      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3695      | MAT α lys2Δ NAAT° ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3696      | MAT α ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |
| TWY3697      | MAT α ura3–52 trp1Δ2 leu2–3112 his3–11 ade2–1 can1–100 elo2Δ::HPH° | This study |

diate Ypk1/2 kinases (18–23).Orm1/2 phosphorylation in turn dissociates the proteins from SPT, relieving their inhibition of the enzyme and increasing SL synthesis. In addition, Ypk1/2 kinases activate ceramide synthase through phosphorylation, thereby catalyzing the subsequent step in SL synthesis (24).

Little is yet known how regulation of different steps in SL synthesis is coordinated. For instance, it is unclear how the activity of the two initial branches of SL biosynthesis, providing sphingoid long-chain bases and VLCFAs, are coordinated. Knowledge of conserved SL regulation mechanisms would enhance our understanding of the regulation of membrane lipids in cells, and could also impact therapies for pathological conditions where SL levels are altered, including the metabolic syndrome and insulin resistance (25) or cancer (26).

Here we utilized a combination of yeast genetics and quantitative mass spectrometry to unravel important regulation of Elo2, one of two elongases catalyzing the first and rate-limiting step of VLCFA synthesis (27, 28). This regulation occurs through Elo2 phosphorylation in a signaling pathway controlled by the guanine nucleotide exchange factor Rom2.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—All yeast strains used in this study are listed in Table 1. All plasmids used are listed in Table 2. ELO2 was cloned in the integrating plasmid pRS306 and Elo2 phosphorylation sites Thr334, Ser336, and Ser338 were mutated to either alanine or aspartate using standard procedures. Plasmids were integrated at the URA3 locus in elo2Δ or elo2Δelo3Δ mutants.

Yeast Culture and Drug Treatment—Yeast cells were grown according to standard procedures in synthetic complete (SC) or rich (YPD) media at 30 °C. Myriocin (Sigma) was dissolved in methanol and used for filter aided sample preparation (30). The resulting peptides were desalted using Sep-Pak C18 cartridges (Waters). The peptide mixture was acidified by addition of TFA and subjected to phosphopeptide enrichment on TiO2 beads as described (29). After elution, the six samples were combined to three samples
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by mixing two fractions. The samples were dried in a SpeedVac to a final volume of 2 ml and buffer A (0.1% formic acid in water) was added to a final volume of 6 ml. 5 ml were analyzed by online nanoflow liquid chromatography tandem mass spectrometry on a Q-Exactive Orbitrap (Thermo) as described previously (31). Raw data were processed by the MaxQuant software package as described previously with the addition of STY phosphorylation as a variable modification (31).

GC/MS—GC/MS samples were prepared as in Ref. 32 and analyzed as described in Ref. 33 with the following modifications. Briefly, cells in midlogarithmic growth phase (−40 A600 units) grown in SC medium were harvested, washed with ice-cold water, and lipids were extracted with methanol:chloroform (1:1). Lipids were dried and re-suspended in 1 ml of 1 M methanolic HCl (Sigma) containing 5% (v/v) 2,2-dimethoxypropane (Sigma). Samples were purged with nitrogen and incubated for 1 h at 80 °C. Samples were cooled to room temperature and fatty acid methyl esters (FAMES) were extracted with hexane, purged with nitrogen, and 2–5 μl were used immediately for injection or stored at −20 °C. GC/MS was performed using an Agilent 7890A GC and Agilent 5975C inert MSD with a J&W DB-XLB column (30 m × 0.18-mm inner diameter, 0.18 m). The acquired spectra were analyzed using Chemstation version A.02.02 software (Agilent). Peak identity was determined using VCLFAME standards (FAME mix C8-C24, methyl heptadecanoate, methyl tricosanoate, Sigma) and predicted using VLCFAME phosphatase inhibitor (04906837001). 30

RESULTS

Components of the Cell Wall Integrity Signaling Pathway Are Required for Cell Growth When Sphingolipid Synthesis Is Compromised—Our previous work generating a genetic interaction map of factors involved in plasma membrane and lipid metabolism revealed an important function for Rom2 in SL regulation (36). Specifically, the genetic interaction pattern for rom2Δ closely resembled those of mutations in early sphingolipid synthesis enzymes, such as Elo2, Sur2, Lcb3, or Elo3 (Fig. 1B) (36). rom2Δ deletion also resulted in synthetic genetic interactions with lcb3Δ, sur2Δ, dpl1Δ and ysr3Δ, all encoding enzymes of SL metabolism. Moreover, compared with wild-type cells, rom2Δ mutants were growth impaired when the SL metabolism was inhibited by myriocin, an SPT inhibitor (Fig. 1C). Based on these findings and the accumulation of sphingoid long-chain bases and concomitant depletion of ceramides in rom2Δ cells (36), we hypothesized that a signaling cascade including Rom2 is important to regulate the abundance of membrane SLs.

Rom2 is a component of the “cell wall integrity pathway” in yeast (Fig. 1C) (37). This signaling pathway orchestrates changes to the cell wall in response to changes in growth state or stresses. In this pathway, Rom2 acts as the GTP exchange factor for the small G-protein Rho1, which in turn regulates the activity of protein kinase C1 (Pkc1) (38–40). Major effects of Pkc1 are mediated by the mitogen-activated protein kinase cascade composed of Bck1, Mkk1, Mkk2 and the mitogen-activated protein (MAP) kinase Slt2.

To test whether Rom2 affects SL metabolism through cell wall integrity signaling, we first tested null mutants of the known sensors of cell wall integrity (Wsc1, Wsc2, Wsc3, Mtl1, and Mid2) (41) in the presence of myriocin and found that wsc1Δ and wsc2Δ cells were sensitive to myriocin. wsc1Δ exhibited the most sensitivity to myriocin, suggesting it is the key sensor in regulating cell wall integrity pathway signaling when sphingolipids are limiting (Fig. 1C).

The next step of the cell wall integrity pathway after the plasma membrane sensors is the activation of Rho1. Because Rom2 activates Rho1 by loading it with GTP (42), we predicted that increasing Rho1 activity should result in the opposite phenotype as displayed by rom2Δ on myriocin containing plates. Consistent with this prediction, deletion of the Sac7 GTPase activating protein (43), which is predicted to increase the amount of active Rho1GTP, led to the suppression of growth inhibition by myriocin (Fig. 1C).

Downstream of Rho1, cell wall integrity signaling continues through a kinase cascade. Consistent with an important role for the initial Pkc1 kinase in maintaining SL levels, cells expressing a constitutive active allele of Pkc1 (pkc1R398P) (40) were able to overcome SL synthesis inhibition by myriocin, growing better

### Table 2: Plasmids used in this study

| Number | Plasmid | Reference |
|--------|---------|-----------|
| TWP55 | pRS416  | 55        |
| TWP124 | CYE67, YCP50, pkc1R398P | 40 |
| TWP317 | pYE323, ypkL259LA | 44 |
| TWP96 | pRS306  | 55        |
| TWP706 | pRS306_ELO2 | This study |
| TWP707 | pRS306 elo2T334A, S336A, S338A | This study |
| TWP708 | pRS306 elo2T334D, S336D, S338D | This study |
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To further investigate the activity of the cell wall integrity signaling pathway under SL-limiting conditions, we hypothesized that the Rom2-dependent signaling cascade regulates SL synthesis. To investigate this, we used phosphoproteomics to identify Rom2-dependent phosphorylation sites (Fig. 2A). To allow for the identification of Rom2-dependent phosphorylation sites that are regulated during sphingolipid depletion, we compared the phosphoproteome of wild-type and rom2 Δ cells after myriocin treatment, which efficiently depletes all SL species (19). Among the 4068 phosphopeptides identified, we found several candidate sites for ROM2-dependent phosphorylation, including proteins with established roles in lipid synthesis (Fig. 2B, supplemental Table S1). Notably, a cluster of two phosphoserines and a phosphothreonine in the C terminus of Elo2 (Thr 334, Ser 336, and Ser 338) contained the most significant outliers of dephosphorylated peptides in rom2 Δ cells (p value < 1E-25; Fig. 2, B and C). We next tested whether these sites were only phosphorylated in myriocin-treated cells or also in basal conditions. Monitoring the three sites by mass spectrometry-based proteomics showed a decrease of all three Elo2 phosphorylation sites in rom2 Δ mutants compared with wild-type cells in the absence of myriocin (Fig. 3, A–C). These data are consistent with previous reports that have identified these three Elo2 sites phosphorylated in exponentially growing wild-type yeast (phosphogrid) (45).

Elo2 is the rate-limiting enzyme in the elongation of fatty acids to VLCFAs. In addition to the decrease in phosphorylation of Elo2, we observed increased phosphorylation of the TORC2 component Bito1 and the TORC2 target Ypk1 after myriocin treatment in cells lacking Rom2 (Fig. 2B). These changes suggest that alterations of SL metabolism modulate TORC2 pathway signaling activity in rom2 Δ cells.

Elo2 Phosphorylation Is Down-regulated during Inhibition of Sphingolipid Synthesis—VLCPAs synthesis is catalyzed by two elongases, Elo2 and Elo3, in yeast. Elo2 prefers shorter chain fatty acids as substrates (e.g. C16, C18) and elongates them to acyl-chains maximally containing 24 carbon atoms (C24). Its homologue Elo3 primarily elongates C24 to C26 fatty acids, which are the major constituents of yeast SLs (28, 34). Consequently, deletion of LO3 results in the complete absence of C26 fatty acids with increased C20–C24 levels, whereas elo Δ mutation results in a significant decrease in all VLCPAs (28). Because Elo3 acts on products of Elo2, we reasoned that Elo2 regulation could control overall VLCPAs for SL synthesis. Consistent with this hypothesis, although Elo3 and Elo2 are highly similar in sequence, Elo3 lacks the putative region of Elo2 that we identified as phosphorylated (Fig. 2D).

than wild-type cells under the same conditions. The extent of this suppression was similar to the effect of expressing a constitutively active version of Ypk2 (ypk2Δ239A) (44), a kinase that up-regulates sphingoid long-chain base synthesis by phosphorylating Orm1/2 proteins (Fig. 1C).

We further systematically tested the effect of the deletion of each downstream kinase of the cell wall integrity signaling cascade on cell growth when SL synthesis is compromised. Deletion of the effector MAP kinase Sl2 led to lethality on myriocin containing plates. Similarly, bck1Δ cells were strongly growth impaired on myriocin containing plates, whereas the effects due to deletion of the genes encoding the redundant Mkk1 or Mkk2 kinases were milder (Fig. 1C).

To further investigate the activity of the cell wall integrity signaling pathway under SL-limiting conditions, we assayed Sl2 activity by monitoring the phosphorylation state of the kinase after myriocin treatment. Interestingly, Sl2 phosphorylation decreased and remained dephosphorylated for the duration of the experiment (Fig. 1D). As a control, we followed the phosphorylation of Orm1. As expected, within 15 min of treatment, an HA-tagged version of Orm1 was phosphorylated.

Rom2-dependent Phosphorylation of the Fatty Acid Elongase Elo2—Based on the data on cell growth under SL limiting conditions, we hypothesized that the Rom2-dependent signaling cascade regulates SL synthesis. To investigate this, we used phosphoproteomics to identify Rom2-dependent phosphorylation sites (Fig. 2A). To allow for the identification of Rom2-dependent phosphorylation sites that are regulated during sphingolipid depletion, we compared the phosphoproteome of wild-type and rom2 Δ cells after myriocin treatment, which efficiently depletes all SL species (19). Among the 4068 phosphopeptides identified, we found several candidate sites for ROM2-dependent phosphorylation, including proteins with established roles in lipid synthesis (Fig. 2B, supplemental Table S1). Notably, a cluster of two phosphoserines and a phosphothreonine in the C terminus of Elo2 (Thr 334, Ser 336, and Ser 338) contained the most significant outliers of dephosphorylated peptides in rom2 Δ cells (p value < 1E-25; Fig. 2, B and C). We next tested whether these sites were only phosphorylated in myriocin-treated cells or also in basal conditions. Monitoring the three sites by mass spectrometry-based proteomics showed a decrease of all three Elo2 phosphorylation sites in rom2 Δ mutants compared with wild-type cells in the absence of myriocin (Fig. 3, A–C). These data are consistent with previous reports that have identified these three Elo2 sites phosphorylated in exponentially growing wild-type yeast (phosphogrid) (45).

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FIGURE 2. Phosphoproteome of Rom2-dependent phosphorylation changes during SL limitation. A, experimental design for phosphoproteomic analysis. Wild-type cells grown in the presence of light lysine and rom2Δ cells grown in the presence of heavy lysine, both treated with myriocin, were used for phosphoproteomic analysis by LC-MS/MS. B, Elo2 phosphorylation is regulated by the cell wall integrity pathway under sphingolipid limited conditions. Heavy/light SILAC ratios of phosphorylated peptides are plotted against peptide intensities. Significant outliers are colored in red (p < 1e-11), orange (p < 1e-4), or light blue (p < 0.05); other proteins are shown in dark blue. Elo2, Ypk1, and Bit61 are highlighted in green. C, mass spectra of Elo2 peptides from light (green) and heavy (red) lysine containing cultures. D, alignment of Elo2 and Elo3 C termini with Elo2 phosphorylation sites Thr334, Ser336, and Ser338 labeled by red boxes.

FIGURE 3. Rom2-dependent phosphorylation of Elo2. A, experimental design for phosphoproteomic analysis. Wild-type cells were grown in the presence of light lysine and rom2Δ cells were grown in the presence of heavy lysine and used for phosphoproteomic analysis by LC-MS/MS. B, log₂-transformed, normalized heavy/light ratios of phosphorylated Elo2 peptides. Down-regulation of Elo2 phosphorylation during SL synthesis inhibition—If VLCFA synthesis is important for maintaining cellular SL homeostasis, we expected a growth phenotype for mutants in the fatty acid elongation cycle under SL limiting conditions. To test this prediction, we serially diluted elo2Δ, elo3Δ, ifa38Δ, tsc13-DAMP, and phs1-DAMP cultures onto plates containing myriocin. We found that each of the mutations suppressed the growth defect due to myriocin (Fig. 5A). Among several possible interpretations, this might indicate that limiting VLCFA synthesis reduces the accumulation of a lipotoxic species accumulating in myriocin-treated cells.

To test whether phosphorylation is important for the physiological function of Elo2 during SL synthesis inhibition, we next assayed mutants in which we replaced each of the co-regulated phosphorylation sites with non-phosphorylatable alanine (elo2AAA) or negatively charged, phosphomimicking aspartate residues (elo2DDD). Among the mutants, elo2DDD cells mimicked the elo2Δ deletion phenotype, rescuing the cells’ growth on myriocin-containing plates, although to a lesser extent than...
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Our findings contrast a recent report that concluded dephosphorylated Elo2 was inactive and rapidly degraded (45). However, C terminally tagged strains of Elo2 used in this earlier study are not appropriately regulated during SL synthesis inhibition (data not shown). Moreover, in constructing ELO2 alleles containing phosphosite mutations, Zimmermann et al. (45) deleted the 3' UTR, which leads to strongly decreased mRNA levels (~70% decreased compared with wild-type, data not shown), similarly to the situation in DAmP alleles (46), likely explaining the observed severe lack of function of the allele.

To further test whether mutation of the Elo2 phosphorylation sites impairs the regulated, but not the basal function of the protein, we assayed the genetic interaction of the mutant with elo3Δ. As expected, the elo3Δelo2Δ double mutation was lethal (47). In contrast, either elo2DDD or elo2AAA supported growth in the absence of Elo3, showing that the mutant proteins maintain at least basal activity in the absence of regulation by phosphorylation (Fig. 5D).

Elo2 Phosphorylation Regulates Very Long-chain Fatty Acid Levels—To test whether Elo2 phosphorylation modulates VLCFA synthesis, we next analyzed the fatty acid composition of Elo2 phosphosite mutants by gas chromatography coupled to mass spectrometry (GC-MS). As expected for the increased activity of dephosphorylated Elo2, cells expressing the elo2AAA mutant showed increased levels specifically of VLCFAs, with the most pronounced effects apparent on C20 to C26 fatty acids (Fig. 6A). Biochemical activity assays with lysates from cells expressing either mutant failed to reveal significant differences in specific VLCFA activity (data not shown), arguing that regulation might occur at the level of substrate binding or localization of the enzymes.

To test whether phosphorylation control is important to regulate VLCFAs during sphingolipid synthesis inhibition, we next tested the effect of the different phosphosite mutants on fatty acid levels during myriocin treatment. We found that wild-type cells that cannot synthesize ceramides due to the limitation of sphingoid long-chain bases during myriocin treatment up-regulate C26 fatty acid. Consistent with an important regulatory function of Elo2 phosphorylation, both elo2AAA and elo2DDD cells failed to respond to the myriocin challenge (Fig. 6B). Together, these data argue that the Elo2 phosphorylation sites are not important for basal activity but to regulate its activity in response to myriocin treatment.

To determine the effects of VLCFA accumulation on the global lipid profile, we next measured the cellular lipidomes of the elo2 phosphosite mutants, elo2Δ, and wild-type strains. In these experiments, total ceramide and complex sphingolipid levels in elo2AAA and elo2DDD were unaltered (Fig. 6C).

If increased VLCFAs do not lead to increased SL levels they should be present in a different lipid pool. To our surprise, the VLCFAs did not enter phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol), but where almost exclusively found in cellular triacylglycerols and sterol esters (Fig. 6C). These findings indicate that cells have the ability to store excess VLCFAs, likely within cytosolic lipid droplets. This increase in triacylglycerols containing VLCFA did not significantly affect total TG content (Fig. 6C) or lipid

the null mutant (Fig. 5, B and C). This phenotype was due to a defect in Elo2 regulation, but not its basal function, as elo2DDD was growth impaired on fenpropimorph, an inhibitor of ergosterol biosynthesis, similar to wild-type cells (Fig. 5, B and C). In contrast, and in agreement with Elo2 activation by dephosphorylation, expression of elo2AAA led to a similar growth phenotype on myriocin containing medium as expression of wild-type Elo2, which is dephosphorylated under these conditions (Fig. 5, B and C).
droplet volume (data not shown). This reflects that even when accumulating in TGs due to a defect in SL synthesis, VLCFAs are still much less abundant than shorter chain fatty acids, such as C18.

Rom2 Regulates Very Long-chain Fatty Acid Levels—Our hypothesis that Rom2-dependent signaling regulates Elo2 predicts that VLCFAs are altered in rom2 Δ cells. Consistent with the model, GC-MS analysis revealed a significant increase in C26 fatty acids in rom2 Δ when shorter VLCFAs did not appear to be affected (Fig. 7A).

We next determined the contribution of Elo2 phosphorylation to Rom2-dependent regulation of SL synthesis. To directly test whether Elo2 phosphorylation was responsible for the VLCFA phenotype of rom2 Δ cells, we measured fatty acid profiles of elo2AAA rom2 Δ and elo2DDD rom2 Δ cells. The phosphomimetic elo2DDD rescued the increase in C26 seen in rom2 Δ alone, whereas the non-phosphorylatable elo2AAA aggravated the VLCFA accumulation. These data further support that Elo2 phosphorylation is a critical control point regulated by Rom2-dependent signaling (Fig. 7B).

If Elo2 is the key downstream effector of Rom2-dependent signaling, the phosphomimetic elo2DDD mutant should rescue the severe growth defect of rom2 Δ cells in the presence of myriocin. If there are other, more critical regulated steps, we expect no significant effect of expressing the phosphomimetic mutants in rom2 Δ cells. Testing these possibilities, we found that elo2DDD significantly decreases the doubling time of rom2 Δ in liquid medium containing a low dose of myriocin. This argues that Elo2 is indeed a critical node of Rom2-dependent SL regulation. However, elo2DDD did not restore the growth rate to that of WT cells, arguing that either the phosphomimetic allele is not fully recapitulating phosphorylation or that additional regulation exists (Fig. 7C).

**DISCUSSION**

Here we identify Elo2 as a key regulation point of VLCFA synthesis. Our findings link the upstream steps of the cell wall integrity signaling cascade to control of Elo2 and VLCFA levels. How does Rom2 regulate Elo2? In a likely scenario, the first phase of signaling during SL limitation diminishes activity of cell wall integrity sensors, such as Wsc1, in the plasma membrane. This in turn down-regulates Rom2 activity toward Rho1 and the downstream signaling cascade. Thus, both Rom2-dependent regulation of VLCFA and TORC2-dependent regulation of sphingosine synthesis respond to changes of the plasma membrane state (12). During myriocin treatment, TORC2 activity is increased, whereas Rom2 activity is decreased. Consistent with this notion, Rom2 localization at the plasma membrane is diminished during myriocin treatment (48). This suggests that cells use the reciprocal regulation of both signaling systems to fine-tune communication on the state of the plasma membrane. Consistent with this notion of two parallel pathways, activation of Rho1 suppresses tor2 mutations (43).

The second phase of signaling, transduction of the signal from the plasma membrane to ER-localized Elo2, is complex. As expected from down-regulating the cell wall integrity pathway, we observed a decrease in phosphorylation of the terminal kinase of the pathway, Slt2. However, we and others did not observe changes in Elo2 phosphorylation in slt2 Δ cells (data not shown) (45). This suggests that the change in Rom2-dependent phosphorylation of Elo2 does not involve the downstream cell wall integrity pathway signaling kinases, such as Slt2. Instead, the signal from RhoGTP may be transduced through another one of its targets, the TORC1 complex (49). Control of TORC1 could relay the information on plasma membrane SL levels to...
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Mck1, a GSK3-like kinase in yeast that mediates Elo2 phosphorylation in the ER (45). Importantly, in other systems, Gsk3 kinases need a priming kinase, which phosphorylates one site of the target, to fully phosphorylate the substrate (50). Thus, it remains possible that yet another kinase downstream of Rom2 is involved and plays this priming function.

Although downstream effectors of the cell wall integrity pathway, such as Slt2, are not required for the change in Elo2 phosphorylation due to myriocin treatment, we find that they are, nonetheless, important for cell growth when sphingolipid synthesis is inhibited. This suggests that the cell wall integrity pathway may have other functions in the homeostatic response regulating plasma membrane composition. Interestingly, rom2Δ has decreased ceramides (36), whereas both long-chain bases and VLCFAs are increased (Fig. 7A) (36), suggesting components of this signaling cascade may be involved in the regulation of ceramide synthase. Another possibility is regulation of the transcription of the Elo2 kinase Mck1 (51).

Our data lead to a model in which Elo2 is regulated by phosphorylation in response to VLCFA levels. First, during times of increased demand for VLCFAs, Elo2 is activated by dephosphorylation. During times of excess VLCFAs levels, Elo2 is deactivated by phosphorylation and VLCFAs are esterified and stored. Rom2 and TORC2 appear to cooperate to provide increased amounts of VLCFA and sphingoid long-chain base substrates for ceramide synthesis. In this model, the function of the Elo2 phosphorylation system is to adjust the flux through the elongation cycle to match the activity of SPT for production of ceramides. In the presence of myriocin, when cells are unable to up-regulate sphingoid long-chain base synthesis through the Orm1/2-system, increased VLCFA pools cannot drive SL synthesis. Instead, in the absence of coordinated regulation of other SL synthesis steps, VLCFAs are shunted toward triacylglycerols and presumably stored in cytosolic lipid droplets. This storage of VLCFAs may reflect a protective mechanism that prevents the build-up of VLCFAs in the ER, which may be lipotoxic. Lipotoxicity could be due to membrane property changes inflicted by sphingolipid synthesis intermediates. Some of the SL synthesis intermediates are also potent signaling molecules. Sphingosine and ceramide, as well as their phosphorylated derivatives, play crucial functions in proliferation control (10). Providing adequate amounts of VLCFAs thus may prevent unintended signaling due to a build-up of SL synthesis intermediates.

Notably, our interpretation that dephosphorylated Elo2 is active is opposite to the interpretation of a recent paper that argued that phosphorylation inactivates Elo2 (45). This discrepancy is likely due to complications of yeast strain construction. Zimmermann et al. (45) generated mutants in the phosphorylation sites at the very C terminus by replacing the sequence encoding the last few amino acids and in the processes deleting the 3' UTR of the corresponding messenger RNA (data not shown). As a consequence, the mRNA transcribed from this allele is strongly reduced, leading to the observed lack of function of this allele. In addition, the C-terminal tags used by Zimmermann et al. (45) next to the phosphorylation site in our hands impair the regulation of Elo2 (data not shown).

The control of Elo2 may be most important when conditions change for the cell. Internal changes of conditions are driven by the cell cycle, for instance. The regulation of the cell wall integ-
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pathway by the cell cycle (37) thus may serve to assure appropriate levels of sphingolipid synthesis when the plasma membrane, containing the bulk of sphingolipids, expands. External changes, such as temperature changes, affect membrane properties, and require a homeostatic response. Consistent with this, the cell wall integrity pathway and sphingolipid synthesis are regulated by heat stress (52).

As salient features of SL metabolism, as well as its control, appear evolutionarily conserved, similar mechanisms of VLCFA regulation might operate in other eukaryotes. Consistent with this notion, human fatty acid elongases, such as ELOVL5, were found to be phosphorylated in systematic analyses (phosida.com). In addition, single point mutations in human ELOVL5 lead to development of neurodegenerative spinocerebellar ataxia (53), suggesting sphingolipid homeostasis regulation may have an important function in the human adult brain. It will be interesting to determine whether ELOVL5 phosphorylation similarly governs VLCFA metabolism in mammalian tissues, and if so, which role this regulation plays in organismal physiology.

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